AD NUMBER

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AUTHORITY

USAMRDC, 04 May 1993

FINAL REPORT

Contract No. DAMD17-83-C-3129
Multiple Animal Studies for Medical Chemical Defense
Program in Soldier/Patient Decontamination and Drug Development

on

TASK ORDER 84-6:
PYRUVATE DEHYDROGENASE SYSTEM FOR DETERMINING THE EFFECTIVENESS OF ARSENIC ANTIDOTES

to

U.S. ARMY MEDICAL RESEARCH
AND DEVELOPMENT COMMAND
INSTITUTE OF CHEMICAL DEFENSE

March 11, 1988

by

Dr. Ronald L. Joiner
Dr. David W. Hobson

BATTELLE
Columbus Division
505 King Avenue
Columbus, Ohio 43201-2693

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SECURITY CLASSIFICATION OF THIS PAGE

ADB120625

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution authorized to U.S. Government agencies and their contractors; Reason - Critical Technology; 3 March 1988.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Battelle Memorial Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION U.S. Army Medical Research Institute of Chemical Defense
6c. ADDRESS (City, State, and ZIP Code) 505 King Avenue Columbus, OH 43201-2693	7b. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5425
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-83-C-3129
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 53751A; PROJECT NO. 3M4-6375D993; TASK NO. BB; WORK UNIT ACCESSION NO. 023

11. TITLE (Include Security Classification)
Multiple Animal Studies for Medical Chemical Defense Program in Soldier/Patient Decontamination and Drug Development

12. PERSONAL AUTHOR(S)
Joiner, Ronald L., Hobson, David W.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 841201 TO 870301	88/03/11	92

16. SUPPLEMENTARY NOTATION
Task Order 84-6: Pyruvate Dehydrogenase System for Determining the Effectiveness of Arsenic Antidotes

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	15	
06	20	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
In vitro screening system - 1. pyruvate dehydrogenase 2. antidote 3. Lewisite 4. Arsenic
(PYRUVATE DEHYDROGENASE)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
An in vitro method for screening arsenic antidotes was developed. This assay uses the inhibition of PDH complex as its basis for quantitation. The assay is effective, inexpensive, and quantitative for comparing the efficacy of reactivators of PDH complex inhibitors which contain As^{+3}. The assay may also be of value in comparing the efficacy of chelating agents for arsenicals containing As^{+5}, and other inhibitors of sulfhydryl-containing enzymes, such as mercury, cadmium, and lead.

KEYWORD; LEWISITE.

Arsenic (3+)

Arsenic (5+)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller	22b. TELEPHONE (Include Area Code) 301-663-7325
	22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FINAL REPORT

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to

U.S. ARMY MEDICAL RESEARCH
AND DEVELOPMENT COMMAND
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March 11, 1988

Ronald L. Joiner, Ph.D.
Study Director

David W. Hobson, Ph.D.
Study Supervisor

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PYRUVATE DEHYDROGENASE SYSTEM FOR DETERMINING THE EFFECTIVENESS OF ARSENIC ANTIDOTES

1.0 INTRODUCTION

An in vitro screening system to evaluate the effectiveness of arsenic antidotes using pyruvate dehydrogenase (PDH) activity as its basis has been established at the Medical Research and Evaluation Facility (MREF) under Task 84-6. Initiated in March 1984, this task developed an in vitro screening system to evaluate the relative effectiveness of candidate antidotal compounds in the prevention and/or reversal of systemic effects resulting from exposure to Lewisite (L) and/or other arsenic-containing compounds.

A research protocol and appropriate safety/surety standard operating procedures (SOPs) were prepared and submitted to the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) of the U.S. Army Medical Research and Development Command (USAMRDC) for comment, modification, and approval. MREF Protocol 16, entitled "Pyruvate Dehydrogenase System for Determining the Effectiveness of Arsenic Antidotes," was signed in October 1984. The protocol was amended twice to clarify procedures described in the original protocol, once in October 1985 and again in October 1986. A copy of the signed research protocol and its amendments is included in Appendix A.

The safety/surety SOP (MREF SOP-83-7), which covers procedures performed during the conduct of the PDH screening test, was prepared in March 1984, revised in August 1984, approved in December 1984, and reviewed for appropriateness in 1985 and 1986. A copy of the signed safety/surety SOP is included in Appendix B.

The PDH complex found in mammalian tissue is a large, multienzyme complex which catalyzes the following overall biochemical reaction:

$$\text{pyruvate} + NAD^+ + \text{CoASH} \longrightarrow \text{acetyl-S-CoA} + CO_2 + \text{NADH}$$

The complex includes three enzymes which catalyze the following individual reactions:

H^+ + pyruvate + TPP -- [E1] --> hydroxyethyl-TPP + CO_2

hydroxyethyl-TPP + $lipS_2$ -- [E2] --> TPP + lip(SH)-S-acetyl

lip(SH)-S-acetyl + CoASH -- [E2] --> $lip(SH)_2$ + acetyl-S-CoA

$lip(SH)_2$ + NAD+ + FAD -- [E3] --> $lipS_2$ + CO_2 + NADH

where:

E1 = pyruvate dehydrogenase
E2 = dihydrolipoyl transacetylase
E3 = dihydrolipoyl dehydrogenase
TPP = thiamine pyrophosphate
NAD = nicotinamide-adenine dinucleotide
FAD = flavin-adenine dinucleotide
$lipS_2$ = lipoic acid
$lip(SH)_2$ = dihydrolipoic acid
CoA = coenzyme A.

Hepatic PDH complex activity is significantly reduced in rats given sodium arsenate (As^{+5}) in their drinking water (Schiller et al., 1977), and sodium arsenite (As^{+3}) inhibits PDH complex activity in mouse kidney extracts in vitro (Hsu et al., 1983). The mechanism by which arsenic inhibits PDH complex activity in mammalian tissues has yet to be fully characterized, but it probably involves arsenic binding to the lipoic acid and dithiol moieties of the complex (Fluharty and Sanadi, 1961). Thus, the reactions mediated by dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase appear to be especially susceptible to the inhibitory action of arsenic and have been used previously as the basis for the development of an in vitro assay to study the efficacy of potential antidotes for arsenic (Hsu et al., 1983; Aposhian et al., 1983).

In the in vitro PDH complex assay scheme used by Hsu et al. (1983), a 3,000 g supernatant from homogenized mouse kidney was the source of PDH complex. Using ^{14}C-pyruvate as the substrate, enzyme kinetic data were derived from the capture of $^{14}CO_2$ evolved from the reaction and its quantitation by liquid scintillation counting. Although this method was shown effective for assaying the inhibitory action of arsenic on PDH complex

activity and its reactivation by a few potential antidotes, the method cannot be seriously considered for routine use as an in vitro screen for arsenic antidotes for several reasons:

(1) The use of PDH complex derived from homogenized mouse kidneys is subject to error due to animal variation and a difficult to control preparatory scheme. It also increases the probability that competing thiol-containing compounds (such as CoASH) may be included in varying amounts in different batches of the crude preparation of PDH complex.

(2) The use of ^{14}C-pyruvate as a substrate is expensive and requires periodic assessment of radiochemical purity and methods for the routine disposal of radioactive waste.

(3) Collection and quantitation of $^{14}CO_2$ requires special reagents and equipment which adds to the expense and difficulty of performing the assay on a routine basis.

(4) $^{14}CO_2$ production occurs in a portion of the catalysis sequence of the PDH complex which is probably only indirectly affected by the inhibitory action of arsenic, i.e., the first reaction, which is catalyzed by PDH. PDH is the only enzyme in the PDH complex which arsenic is not thought to directly inhibit.

Therefore, Battelle proposed to establish an in vitro screening test procedure at the MRFF to determine the effectiveness of antidotes for arsenic which would use purified PDH complex obtained from a commercial source and in which the overall catalytic activity of the complex would be determined spectrophotometrically by monitoring the rate of NADH production. Once established, the assay would be used to determine: (1) whether toxic, organic arsenicals, such as L and chlorovinylarsenous acid (CVAA), also produce inhibition of the PDH complex, and (2) whether the antidotal activity of therapeutic agents such as 2,3-dimercaptopropanol (BAL), meso-2,3-dimercaptosuccinic acid (DMSA), and 2,3-dimercapto-1-propanesulfonic acid (DMPS) could be effectively evaluated with the assay.

2.0 EXPERIMENTAL DESIGN

2.1 REAGENTS AND MATERIALS

The following reagents and test articles were used in the establishment of the in vitro screening system for arsenic antidotes using PDH complex as described in MREF Protocol 16:

- Purified PDH complex derived from bovine cardiac muscle - Sigma Chemical Company (St. Louis, MO 63178; stock number P-6152). All assays were performed using PDH complex from lot number 36F-8055. Characterization of this lot, as reported by the manufacturer, was as follows: protein (Lowry method) = 2.7 mg/mℓ, PDH complex activity = 4.0 units/mg of protein (unit = 1.0 μM of NAD converted to NADH per min in the presence of saturating levels of coenzyme A at pH = 7.4 and 30 C).
- Sodium arsenite (>99.9 percent purity, lot KJSA) - Mallinckrodt, Inc. (Paris, KY 40361).
- L (dichloro-2-chlorovinylarsine, 98 percent pure, lot number 39135-4) - USAMRICD.
- CVAA - prepared by hydrolysis of L using a procedure (Appendix F) supplied by USAMRICD.
- 2,3-Dimercaptopropanol (BAL, stock number D-1129, lot number 104F-0409) - Sigma Chemical Company.
- 2,3-Dimercapto-1-propanesulfonic acid (DMPS, stock number D-8016, lot number 92F-0337) - Sigma Chemical Company.
- Meso-2,3-dimercapto-succinic acid (DMSA, stock number D-7881, lot number 114F-0377) Sigma Chemical Company.
- Sodium pyruvate (99 percent pure, stock number P-2256, lot number 63F-0493) - Sigma Chemical Company.
- DL-Cysteine, HCl (stock number C-9768, lot number 64F-0260) - Sigma Chemical Company.
- Coenzyme A (CoA, 96 percent pure; stock number C-3144, lot number 86F-7181) - Sigma Chemical Company.

- Nicotinamide adenine dinucleotide (NAD, 99 percent pure, stock number N-1636, lot number 125F-7085) - Sigma Chemical Company.
- Thiamine pyrophosphate (TPP, 97 percent pure, stock number C-8754, lot number 34F-0674) - Sigma Chemical Company.
- Ethylenediaminetetraacetic acid (EDTA, 99 percent pure, stock number ED2SS, lot number 14F-0027) - Sigma Chemical Company.
- Calcium chloride (CaCl2, 99 percent pure, stock number C-3881, lot number 45F-0121) - Sigma Chemical Company.
- Magnesium chloride (MgCl2, 98 percent pure, stock number M-8266, lot number 36F-3490) - Sigma Chemical Company.
- Tris(hydroxymethyl)aminomethane (TRIS, 99 percent pure, stock number T-3253, lot number 96F-5639) - Sigma Chemical Company.

A description of all reagents and amounts used in the preparation of stock solutions, which were required in the conduct of all assays to determine PDH complex activity, can be found in Appendix A.

Spectrophotometric measurements were taken at 340 nm using a Hewlett-Packard (Palo Alto, CA 94304) model 8451A diode array spectrophotometer (2-nm bandwidth, 1-second integration time) equipped with a thermostatable cell holder set at 30 C.

A Mettler (Hightstown, NJ 08520) AE-163 analytical balance was used to weigh all reagents, and pH determinations were made using either a Beckman-Altek (Fullerton, CA 92634) model theta 70 or a Corning (Corning, NY 14830) model 140 pH meter.

2.2 ASSAY OF PDH COMPLEX ACTIVITY

The substrate and buffer systems selected for use in the establishment of the PDH reactivator/inhibitor screening assay were essentially those of Hsu et al. (1983). These systems were selected because they had been previously used with good success to examine the effectiveness of potential arsenic antidotes using mammalian PDH complex derived from mouse kidney homogenates.

The overall PDH complex screening assay developed for use in the present study did, however, differ from the assay described previously by Hsu et al. (1983) in a few significant respects. First, the activity of the PDH complex was determined spectrophotometrically by measuring the rate of increase in absorption at 340 nm associated with the conversion of NAD to NADH by dihydrolipoyl dehydrogenase. In the previously described assay, the amount of ^{14}C-pyruvate converted to $^{14}CO_2$ after 10 min in the presence of PDH complex and cofactor mixture was used as the index of PDH complex activity. Second, the volumes of cofactor matrix and substrate used were adjusted to allow reading of the reaction mixture in a disposable cuvette with a 1-cm path length. Third, the substrate, sodium pyruvate, was added to the PDH complex/cofactor solution after incubation in the presence of inhibitor for 10 min at 30 C, not at the same time that inhibitor was added. Finally, the concentration of PDH complex used was determined experimentally, rather than by attempting to add an amount of activity equivalent to that used by Hsu et al. (1983). The concentration of PDH complex used was selected in order to permit inhibition of the complex by concentrations of L and CVAA well below designated surety levels and to maximize the number of assays that could be performed using a single 2- to 3-mℓ vial of commercially prepared PDH complex. A complete description of PDH complex assay parameters used in this study is given in Appendix A.

A typical curve showing the rate of conversion of NAD to NADH by commercially-supplied PDH complex under assay conditions is given in Figure 2.1. Due to the linearity of the absorbance values, the period between 3 and 6 min after addition of sodium pyruvate to the PDH complex/cofactor mixture was selected for estimation of reaction rate.

Reaction rate for each assay was determined by linear regression using the absorbance values obtained between 3 and 6 min after addition of the substrate. This rate value was then used to calculate the units/liter (U/ℓ) activity of PDH complex in the reaction mixture, using the following equation:

$$\text{PDH activity (U/ℓ)} = \frac{dA}{\text{min}} \times \frac{1}{6.22} \times \frac{\text{total volume}}{\text{sample volume}} \times 1000$$

where:

dA/min = change in absorbance at 340 nm per min

6.22 = millimolar (mM) absorptivity of NADH at 340 nm ($mM^{-1} \times cm^{-1}$)

total volume = total volume of material in cuvette (1.045 mℓ)

sample volume = volume of enzyme sample added to cuvette (0.05 mℓ)

1000 = factor required to convert mM value to micromolar (μM) value.

Therefore:

$$\text{PDH activity (U/ℓ)} = \frac{dA}{min} \times 3360.129 \quad .$$

During the day-to-day conduct of the screening assay in the laboratory, a rough estimate of dA/min was obtained by two point subtraction [dA/min = (6-min absorption value - 3-min absorption value)/3]. This estimate was used for daily assessment of assay results and to quickly determine the status of individual samples as they were assayed.

Final values for dA/min and PDH complex activity were calculated in the computer program found in Appendix E. This program also provided sample percent activity and percent inhibition relative to an appropriate control.

2.3 CONDUCT OF PDH COMPLEX INHIBITION/REACTIVATION ASSAYS

Inhibition assays were conducted with each candidate inhibitor of PDH complex activity for two purposes: (1) to determine whether the agent was an inhibitor of PDH complex activity as estimated by the NAD to NADH conversion assay, and (2) to establish the inhibitor concentration required to produce approximately 90 percent inhibition of the PDH complex activity in a control sample. This target inhibition level was selected because values of 90 percent inhibition were found experimentally to be less difficult to attain (in the case of sodium arsenite) or to estimate (in the cases of L and CVAA) than an inhibition value of 100 percent. In addition, the 90 percent value permitted a ready means by which possible problems associated with the accidental addition of excess inhibitor or loss of inhibitor potency could be quickly identified on a per sample basis. Concentrations of inhibitors selected for routine use in screening assays were based on inhibition values obtained using two point

subtraction estimates of dA/min. The use of this procedure allowed work to progress smoothly from one day of assays to the next without delay. Final values for inhibitor assays were calculated using the computerized procedure described above and were used in all subsequent statistical procedures involving inhibition values.

Candidate reactivators were evaluated by performing a standard set of assays for each reactivator concentration level. A set of assays consisted of the following samples: an enzyme control, an inhibitor-only sample, a reactivator-only sample, and up to 5 inhibitor + reactivator samples. Percent PDH complex activity remaining following each treatment was computed on a per set basis in the following formulae:

Enzyme control = 100 percent (arbitrary fixed value for each set of samples).

$$\text{Inhibitor-only \%} = \frac{\text{Inhibitor-only dA/min}}{\text{Control dA/min}} \times 100$$

$$\text{Reactivator-only \%} = \frac{\text{Reactivator-only dA/min}}{\text{Control dA/min}} \times 100$$

$$\text{Inhibitor + Reactivator \%} = \frac{\text{Inhibitor + Reactivator dA/min}}{\text{Reactivator-only dA/min}} \times 100$$

The percent activities of reactivator + inhibitor samples were computed using the activity of the reactivator-only sample in each set for comparison in order to block out any effects on PDH complex activity due to the reactivator alone. This was necessary because several DMPS and DMSA reactivator-only samples exhibited PDH complex activities slightly in excess of their respective control samples.

Reactivator concentrations selected for evaluation were the following reactivator/inhibitor molar ratios:

Inhibitor	Molar Ratios of Reactivator/Inhibitor Tested
Sodium arsenite	1, 1.2, 1.5, 2, 3, 4
L	1, 1.5, 2, 3, 5
CVAA	1, 2, 3, 4, 5 (7, 9 = DMSA only)

A reactivator/inhibitor ratio of 5 was arbitrarily selected as an upper limit value in the screening procedure (except for sodium arsenite in which a value of 5 began to exceed the solubility limits of BAL and DMSA in the TRIS buffer). A reactivator/inhibitor ratio of 1 was arbitrarily selected as the lower limit for evaluation.

The order of evaluation of reactivator effectiveness began with the highest and lowest reactivator/inhibitor ratios, followed by 2 or 3 additional ratios selected on an individual basis following an examination of previously collected data. Under standard assay conditions, as described in Appendix A, the incubation time for inhibitor-only samples was 10 min prior to the addition of substrate. With reactivator/inhibitor samples, reactivator was added 5 min after the addition of inhibitor and the reactivator/inhibitor combination was then allowed to incubate for 5 min in the presence of PDH complex before the addition of substrate. Control samples were incubated for 10 min, and deionized water was added in place of inhibitor and reactivator. Reactivator control samples had deionized water added in place of inhibitor and 5 min later, reactivator was added to the sample and allowed to incubate for 5 min prior to the addition of substrate.

Finally, in order to determine whether length of exposure to inhibitor could be considered a relevant factor in the PDH complex reactivation process, a concentration of DMPS, which was previously observed to be only partially effective when added 5 min after the addition of a PDH complex inhibitor, was added to samples containing each of the three inhibitors after 1 min. The samples were then allowed to incubate for 5 min before substrate was added. Statistical comparisons between the percent PDH complex activity values following the addition of DMPS after 1 min and corresponding data for the addition of DMPS after 5 min incubation were used as the basis for conclusions.

2.4 STATISTICAL ANALYSIS

After collection of all experimental data for inhibitor and reactivator/inhibitor assays, equations of best fit were estimated for each inhibitor or reactivator/inhibitor combination, using the NLIN procedure of the

Statistical Analysis System (SAS, version VMS 3.X, release 4.10) program (SAS Institute, Inc., Cary, NC). The type of equation required for best fit to the data was found to vary according to the type of assay.

Inverse exponential regression curves were computed for inhibition assays, using relative PDH complex activity as a function of inhibitor concentration for each of the three inhibitors examined. The general equation fitted was of the form:

$$A = B_0 + B_1C + B_2e^{(B_3C)}$$

where:

A = percent PDH complex activity

C = inhibitor concentration (mM)

$B_0 \ldots B_3$ = fitted regression parameters.

Quartic polynomial regression equations of best fit to data were computed for reactivator/inhibitor assays, using relative PDH complex activity as a function of reactivator/inhibitor ratio for each of the 9 reactivator/inhibitor combinations examined. These equations were used to predict the lowest reactivator/inhibitor molar ratios required for 100 percent reactivation of PDH complex activity. Because the 100 percent molar ratio could not be used for meaningful statistical comparisons between predicted values, similar estimates of the molar ratios and their associated standard deviations for 90 percent reactivation of PDH complex activity were used instead. This 90 percent value should be of greater predictive value for candidate reactivator selection than the ED_{50} value used previously by Hsu et al. (1983), because greatest separation of the molar ratios required for PDH complex reactivation generally occurs nearer the 100 percent region rather than around the 50 percent values. ED_{50} estimation procedures provide greatest predictive certainty in the 50 percent region and very little certainty in the 100 percent region. By employing the quartic polynomial regression procedure, predictive uncertainty about the 100 percent value is reduced significantly because all of the experimental data are used to develop an equation whose

parameters best fit the experimental observations. In developing the quartic regression models used here, the NLIN procedure of the SAS program was used to compute the estimates of best fit to the following equation:

$$A = B_0 + B_1R + B_2R^2 + B_3R^3 + B_4R^4$$

where:

A = percent PDH complex activity

R = reactivator/inhibitor molar ratio

$B_0 \ldots B_4$ = fitted regression parameters.

All data relating to reactivation of PDH complex activity for a given inhibitor were statistically examined to determine which reactivator/inhibitor combination was most effective at each molar ratio selected. Statistically significant ($P \leq 0.05$) differences between different reactivator/inhibitor combinations were detected using analysis of variance (ANOVA), followed by a studentized range test (Tukey, 1953) to control experiment-wise error rate and a least squares means comparison to control comparison-wise error rate. Statistical comparisons of molar ratio estimates, obtained from quartic polynomial regression equations, for 90 percent PDH complex reactivation were performed as follows:

(1) Obtain quartic parameter estimate of the predicted molar ratio at 90 percent for each reactivator/inhibitor combination.

(2) Determine the 95 percent confidence interval around the above ratio by interpolation and then calculate the standard deviation for the ratio.

(3) Perform an F test comparing all reactivator pairs for each inhibitor to obtain the degrees of freedom for t.

(4) Perform a t-test using the df obtained above (2-sided, alpha = 0.02) to control for experiment-wise error at alpha = 0.05 using Bonferroni's correction (Miller, 1981) for simultaneity of tests).

Student's t-test (alpha = 0.05) was used to compare means from DMPS reactivation of PDH complex activity after 1 min and 5 min of incubation with each of the 3 inhibitors examined.

3.0 RESULTS

Tables are presented in Appendix C and Figures are presented in Appendix D.

3.1 INHIBITION OF PDH COMPLEX ACTIVITY BY SODIUM ARSENITE

Data obtained from PDH complex inhibition assays using sodium arsenite serial dilutions in deionized water (pH = 8) are presented in Figure 3.1. The inverse exponential regression model of best fit to the experimental data was described by the following equation:

$$A = 30.343 + (-2.235 * C) + 73.556 * e^{(-1.579 * C)}$$

where:

A = percent PDH complex activity

C = inhibitor concentration (mM).

The actual changes in absorbance/min, calculated U/ℓ activity, and percent activity values obtained for sodium arsenite are given in Table 3.1. A stock 5 mM concentration of sodium arsenite, based on the percent activity values obtained from different stock concentrations, was selected for routine use in the conduct of reactivator effectiveness assays.

3.2 INHIBITION OF PDH COMPLEX ACTIVITY BY L

Data obtained from PDH complex inhibition assays using serial dilutions of L in TRIS buffer (pH = 8) are presented in Figure 3.2. The inverse exponential regression curve of best fit to the experimental data was described by the following equation:

$$A = 3.067 + (-0.390 * C) + 93.337 * e^{(-48.814 * C)}$$

where:

A = percent PDH complex activity

C = inhibitor concentration (mM).

Actual changes in absorbance/min, calculated U/ℓ activity, and percent activity values obtained for PDH complex inhibition by L are given in Table 3.2. A 0.1 mM stock concentration of L was selected for routine use in the conduct of reactivator effectiveness assays.

3.3 INHIBITION OF PDH COMPLEX ACTIVITY BY CVAA

Data obtained from PDH complex inhibition assays using CVAA serial dilutions in TRIS buffer (pH = 8) are presented in Figure 3.3. The inverse exponential regression curve of best fit to the experimental data was described by the following equation:

$$A = 6.214 + (-16.506 * C) + 95.361 * e^{(-81.923 * C)}$$

where:

A = percent PDH complex activity

C = inhibitor concentration (mM).

Actual changes in absorbance/min, calculated U/ℓ activity, and percent activity values obtained for PDH complex inhibition by CVAA are given in Table 3.3. A 0.2 mM stock concentration of CVAA was selected as the concentration for routine use in the conduct of reactivator effectiveness assays.

3.4 COMPARISON OF PDH COMPLEX INHIBITION BY SODIUM ARSENITE, L, AND CVAA

Figure 3.4 compares the PDH complex inhibition curves for sodium arsenite, L, and CVAA. Sodium arsenite is a much less potent inhibitor of PDH complex activity than either L or CVAA, requiring approximately 25 to 50 times more arsenic in the form of sodium arsenite to produce PDH complex inhibition equivalent to that produced by either CVAA or L. This is true even though arsenic is present in the more toxic +3 valence state in all 3 compounds.

L was as potent a PDH complex inhibitor as CVAA. The slight difference between their inhibition curves is probably attributable to small stoichiometric differences in the amount of active hydrolysis products in their

respective stock solutions. These differences probably result from the fact that CVAA was prepared by hydrolysis of L at pH values equal to or less than 5.8, whereas L stock was prepared by direct addition of L to pH = 8 TRIS buffer. It has been reported previously that L hydrolysis products and their relative amounts vary significantly with pH (Waters and Williams, 1950).

3.5 REACTIVATION OF SODIUM ARSENITE INHIBITION OF PDH COMPLEX ACTIVITY BY BAL, DMPS, OR DMSA

Figures 3.5.1, 3.5.2, and 3.5.3 show percent PDH complex activity as a function of each of the BAL, DMPS, or DMSA/sodium arsenite combinations examined, respectively. Values are given as means and standard deviations expressed as a percentage of the total PDH complex activity of the paired reactivator control sample for each reactivator concentration tested. Figure 3.5.4 compares the mean PDH complex activity values obtained for BAL, DMPS, and DMSA at each molar ratio tested, using a smoothed spline curve through the individual reactivator means. At each molar ratio tested, statistical ($P \leq 0.05$) comparisons of the mean percent activity values obtained for each reactivator resulted in the following rankings:

Molar Ratio Tested	Statistical Ranking of Reactivator Means[1]		
	BAL vs DMPS	BAL vs DMSA	DMPS vs DMSA
1.0	>	>	>
1.2	=	=	=
1.5	<	>	>
2.0	=	=	=
3.0	=	<	<
4.0	<	<	<

[1] Symbols (>,=,<) indicate the statistical relationship between the first vs the second reactivator being compared at $P \leq 0.05$.

At molar ratio = 2, all three reactivators examined had reached the 100 percent activity target for PDH complex reactivation. Above this value, differences between reactivation means were not large and statistically

significant differences were not considered to be related to differences in the ability of the reactivator to restore the PDH complex activity from sodium arsenite inhibition.

Variation in the percent PDH complex activity values for the 20 interassay sodium arsenite inhibitor controls was small (mean = 9.1 percent, S.D. = 3.3 percent). Thus, differences observed in reactivation means were most likely the result of actual differences in reactivator activity.

The best fit quartic polynomial estimates, used to predict the lowest molar ratios required for 100 percent reactivation and for estimation of the molar ratios and their standard deviations for 90 percent reactivation of PDH complex activity by each of the three reactivators tested were as follows:

Reactivator	B^0	B^1	B^2	B^3	B^4
BAL	-348.401	697.479	-384.751	89.590	-7.548
DMPS	-986.905	1,972.066	-1,261.62	338.254	-32.307
DMSA	-641.822	1,215.807	-721.569	184.179	-17.033

The predicted lowest molar ratios of BAL, DMPS, or DMSA to sodium arsenite required for 100 percent reactivation of PDH complex activity, using their respective best fit quartic polynomial estimates, were BAL = 1.597, DMPS = 1.340, and DMSA = 1.625.

Statistical comparison of the estimated molar ratios of each reactivator for 90 percent reactivation of PDH complex activity resulted in the following mean (S.D.) values and rankings:

Reactivator	90% Molar Ratio	Comparison	Significance[1,2]
BAL	1.388(0.128)	BAL vs DMPS	* (>)
DMPS	1.259(0.066)	DMPS vs DMSA	* (<)
DMSA	1.448(0.158)	DMSA vs BAL	ns

1 * = significantly different, or ns = not significantly different at $P \leq 0.05$.
2 The symbols < or > indicate the significant relationship between the first and second member of the reactivators being compared.

3.6 REACTION OF L INHIBITION OF PDH COMPLEX ACTIVITY BY BAL, DMPS, OR DMSA

Figures 3.6.1, 3.6.2, and 3.6.3 show percent PDH complex activity as a function of each of the respective BAL, DMPS, or DMSA/L combinations examined. Data are presented as means and standard deviations expressed as a percentage of the total PDH complex activity of the paired reactivator control sample for each reactivator concentration tested. Figure 3.6.4 compares the mean PDH complex activity values obtained for BAL, DMPS, and DMSA at each molar ratio tested using a smoothed spline function to connect the individual reactivator means.

At each molar ratio, statistical ($P \leq 0.05$) comparisons of the mean percent activity values obtained for each reactivator resulted in the following rankings:

Molar Ratio Tested	Statistical Ranking of Reactivator Means[1]		
	BAL vs DMPS	BAL vs DMSA	DMPS vs DMSA
1.0	>	>	>
1.5	=	=	>
2.0	=	>	>
3.0	=	>	>
5.0	>	=	=

[1]Symbols (>,=,<) indicate the statistical relationship between the first and the second reactivator being compared at $P \leq 0.05$.

BAL, DMPS, and DMSA achieved the 100 percent reactivation activity target for PDH complex reactivation at experimental reactivator/inhibitor molar ratios of 2, 3, and 5, respectively. Differences between percent reactivation means were generally large for BAL vs DMSA and DMPS vs DMSA at molar ratios of 3 and below. At molar ratio = 5, differences between reactivators were not considered to be related to differences in the ability of the reactivator to restore PDH complex activity from L inhibition.

Variation in the percent PDH complex activity values for the 18 interassay L (0.1 mM) controls was small (mean = 11.1 percent, S.D. = 3.1 percent). Thus, differences observed in reactivation means were most likely the result of actual differences in reactivator activity.

The best fit quartic polynomial estimates were as follows:

Reactivator	B^0	B^1	B^2	B^3	B^4
BAL	652.112	-1,377.770	1,015.268	-285.265	26.609
DMPS	182.881	-408.794	339.717	-96.943	8.915
DMSA	-48.304	94.605	-50.513	17.420	-1.984

The predicted lowest molar ratios of BAL, DMPS, or DMSA to L for 100 percent reactivation using their respective best fit quartic polynomial estimates were BAL = 1.989, DMPS = 2.129, and DMSA = 3.201.

Statistical comparison of the estimated molar ratios of each reactivator for 90 percent reactivation of PDH complex activity resulted in the following values and rankings:

Reactivator	90% Molar Ratio	Comparison	Significance[1]
BAL	1.904(0.158)	BAL vs DMPS	ns
DMPS	1.983(0.191)	DMPS vs DMSA	ns
DMSA	2.987(2.201)	DMSA vs BAL	ns

[1] ns = not significantly different at $P \leq 0.05$.

3.7 REACTIVATION OF CVAA INHIBITION OF PDH COMPLEX ACTIVITY BY BAL, DMPS, OR DMSA

Figures 3.7.1, 3.7.2, and 3.7.3 show percent PDH complex activity as a function of each of the BAL, DMPS, or DMSA/CVAA combinations examined, respectively. Values are presented as means and standard deviations expressed as a percentage of the total PDH complex activity of the paired reactivator control sample for each reactivator concentration tested. Figure 3.7.4 compares the mean PDH complex activity values obtained for BAL, DMPS, and DMSA at each molar ratio tested using a spline function to connect the means from each reactivator.

At each molar ratio, statistical ($P \leq 0.05$) comparisons of the mean percent activity values obtained for each reactivator resulted in the following rankings:

Molar Ratio Tested	Statistical Ranking of Reactivator Means[1,2] BAL vs DMPS	BAL vs DMSA	DMPS vs DMSA
1.0	=	=	=
2.0	>	>	<
3.0	>	>	>
4.0	=	>	>
5.0	<	>	>

[1] Symbols (>,=,<) indicate the statistical relationship between the first and the second reactivator being compared at $P \leq 0.05$.
[2] DMSA reactivation only was tested at molar ratios of 7.0 and 9.0 and cannot, therefore, be included here for comparison with BAL or DMSA results at the same molar ratios.

At molar ratio = 5, DMSA still had not reached the 100 percent activity target for PDH complex reactivation. Therefore, molar ratio values of 7 and 9 were included for DMSA to obtain the data necessary to estimate the molar ratio required for 90 percent reactivation and the lowest molar ratio required for 100 percent reactivation of the PDH complex. BAL and DMPS both had attained 100 percent reactivation by molar ratio = 3; above this value, differences between reactivation means between BAL and DMPS were not large, and statistically significant differences were not considered to be related to differences in the ability of the reactivator to restore the PDH complex activity from inhibition by CVAA.

Variation in the percent PDH complex activity values for the 24 interassay CVAA controls used was greater than that observed previously with sodium arsenite or L, but was still within acceptable limits (mean = 9.3 percent, S.D. = 6.4 percent). Therefore, the observed differences in reactivation means are most likely the result of actual differences in reactivator activity.

The best fit quartic polynomial estimates were as follows:

Reactivator	B^0	B^1	B^2	B^3	B^4
BAL	459.925	-926.327	614.514	-151.585	12.574
DMPS	311.525	-574.789	341.053	-74.748	5.584
DMSA	-23.366	34.658	-2.952	0.087	-0.002

The predicted lowest molar ratios of BAL, DMPS, or DMSA to CVAA predicted for 100 percent reactivation using their respective best fit quadratic polynomial estimates were BAL = 2.421, DMPS = 3.182, and DMSA = 7.250.

Statistical comparison of the estimated molar ratios of each reactivator for 90 percent reactivation of PDH complex activity resulted in the following values and rankings:

Reactivator	90% Molar Ratio	Comparison	Significance[1,2]
BAL	2.322(0.160)	BAL vs DMPS	* (<)
DMPS	2.987(0.255)	DMPS vs DMSA	* (<)
DMSA	5.445(2.299)	DMSA vs BAL	* (>)

1 * = significantly different at $P \leq 0.05$.
2 The symbol < or > indicates the significant relationship between the first and second member of the reactivators being compared.

3.8 COMPARISON OF PDH COMPLEX REACTIVATION FROM SODIUM ARSENITE, L, OR CVAA INHIBITION AFTER THE ADDITION OF DMPS AT 1 OR 5 MINUTES AFTER INHIBITION

The protocol used for the routine evaluation of PDH complex reactivator/inhibitor combinations called for reactivators to be added to the enzyme assay mixture following 5 min incubation with inhibitor. The resulting reactivator/inhibitor combination was then incubated for an additional

5 min prior to the addition of substrate. The effect of time of addition on reactivator effectiveness was examined in a series of assays performed with a single concentration of DMPS per inhibitor (identical to the previous assays except that DMPS was added 1 min after the addition of inhibitor). The combined mixture was then incubated for 5 min. A statistical comparison of the mean PDH complex percent reactivation values obtained after 1-min incubation vs. the corresponding previous 5-min value was performed using a t-test. The results were as follows:

Inhibitor Tested	DMPS (mM) (R/I ratio)	Incubation Time (min)	% PDH Complex[1] Reactivation	Significance[2]
Sodium arsenite	5(1)	1	29.15(2.24)	
	5(1)	5	29.30(3.14)	ns
L	0.1(1)	1	58.23(2.73)	
L	0.1(1)	5	22.78(1.09)	*
CVAA	0.2(2)	1	31.68(2.24)	
CVAA	0.2(2)	5	17.53(2.78)	*

1 Values are given as mean (standard deviation).
2 * = The mean 1 min percent reactivation value was significantly different ($P \leq 0.01$) from the corresponding 5-min value.
ns = Percent reactivation values were not significantly different ($P \leq 0.05$).
R/I = reactivator/inhibitor.

4.0 DISCUSSION

4.1 INHIBITION OF PDH COMPLEX ACTIVITY

The inhibition of commercially supplied PDH complex by sodium arsenite was shown to be qualitatively similar to the inhibition of PDH complex derived from mouse kidney homogenates reported previously by Hsu et al. (1983)

and Aposhian et al. (1983). Inhibition curves were similar in shape, including the fact that 100 percent inhibition of PDH complex activity was as difficult to attain with sodium arsenite in this study as in previous studies. Although the per sample concentration of sodium arsenite required for 90 percent inhibition of PDH complex activity was 225 ($\pm$25) μM for both assays, the uncertain purity of the PDH complex preparation used by previous investigators makes this similarity between findings less clear.

L and CVAA were more potent inhibitors (about 25 times for L and about 50 times for CVAA) of PDH complex activity than was sodium arsenite. The reason for this cannot be related to the nature of the arsenic species present in the compounds, since arsenic is in the +3 valence state in all three. The concentration of PDH complex in each assay was constant, as were the concentrations of all required cofactors. It would appear that the binding of As^{+3} to the covalently bound lipoic acid substrate of dihydrolipoyl transacetylase is greater per mole of L and CVAA than for sodium arsenite. This is possible because the arsenite ion (AsO_2^-) provides only one readily available binding site for interaction with the thiol moieties of lipoic acid, whereas L and CVAA will each provide two binding sites, one from each hydrolyzed chloro group in the case of L and one from each hydroxyl group in the case of CVAA. Dithiol binding probably produces greater inhibition of PDH activity than monothiol binding because dithiol binding is more difficult to remove from the enzyme active site and has the additional feature of blocking binding of the substrate to the enzyme active site by tying up several substrate molecules per As^{+3} molecule. Dithiol binding is also supported by the fact that the length of time the PDH complex was exposed to L and CVAA was significant, but for sodium arsenite not significant. In addition, the organic constituents of L and CVAA may cause these materials to be more soluble in the PDH enzyme complex, thereby giving them greater access to the active regions. Furthermore, the organic portions of the L and CVAA molecules may also interfere with substrate binding due to steric hindrance from their presence on the substrate alone. Nevertheless, the results obtained do indicate that L and CVAA are extremely potent inhibitors of PDH complex activity, a finding which is reported here for the first time.

The precise reason for the differences observed in the inhibitory actions of sodium arsenite and the two organic arsenical compounds tested probably involves a combination of the above mentioned factors. It would be of interest to see the degree to which a secondary, R-substituted As^{+3} compound, such as dimethylarsenic acid (DMAA), would inhibit PDH complex activity. Based on the findings from this study, one might predict that DMAA inhibition would fall somewhere between sodium arsenite and either L or CVAA.

Further work is required to better understand the mechanism behind the observed differences in PDH complex inhibition between the three compounds examined in this study. The assay method developed would be useful in this regard.

4.2 REACTIVATION OF PDH COMPLEX INHIBITION BY SODIUM ARSENITE, L, OR CVAA USING BAL, DMPS, OR DMSA

DMPS (Hsu et al., 1983) and DMSA (Aposhian et al., 1983) can reactivate PDH complex inhibition by sodium arsenite. This study further shows that BAL is also a very effective reactivator of sodium arsenite-inhibited PDH complex activity. The estimated molar ratios of each reactivator to sodium arsenite for 90 percent reactivation indicated that both DMPS and BAL are much better reactivators of PDH complex activity than DMSA. DMPS appears to be only slightly better than BAL in the restoration of PDH complex activity from inhibition by sodium arsenite. At the lower reactivator/sodium arsenite ratios tested, differences in effectiveness are diminished between all three reactivators, but DMPS and BAL still appear to be slightly more effective reactivators than DMSA.

The DMPS/sodium arsenite molar ratio estimated by Hsu et al. (1983) to be required for complete reversal of PDH complex inhibition was 2.0. The lowest DMPS/sodium arsenite molar ratio for 100 percent reactivation of PDH inhibition estimated from the results obtained in the present study is 1.34. The results obtained here appear to agree with the previous results of Hsu et al. (1983), especially when the relative differences in the sources and purity of PDH complex used in the two studies are considered.

All three reactivators tested were able to completely reverse PDH complex inhibition by L or CVAA. The relative reactivator/inhibitor molar ratios obtained indicated that BAL and DMPS were more effective reactivators than DMSA against L or CVAA. BAL also appeared to be slightly better than DMPS in this regard. The molar ratio estimates for 100 percent PDH complex reactivation were somewhat greater for each reactivator tested against L or CVAA than those observed for sodium arsenite. This difference may be related to the apparent differences in the mechanism of PDH complex inhibition between the organic arsenicals and sodium arsenite described above.

Reactivation of PDH complex activity by DMPS after exposure to either L or CVAA for 1 or 5 min indicated that time of exposure can significantly affect the percent reactivation value obtained with the PDH complex assay. The effect of 1- or 5-min exposure to sodium arsenite on PDH complex reactivation by DMPS was not significant. Other variables that were controlled in this study, but may significantly affect the assay results, are pH and temperature of the reaction mixture, differing concentrations and ratios of the required cofactors, and the rate of reaction between inhibitor and reactivator.

Overall, the relative ranking of the reactivators tested against all three PDH complex inhibitors used in this study is:

BAL>DMPS>>DMSA

The true relevance of these _in vitro_ findings to _in vivo_ effects cannot be determined without conducting proper validation studies. Hsu et al. (1983) and Aposhian et al. (1983) have shown good correlation between _in vitro_ and _in vivo_ results for reactivation of sodium arsenite by either DMPS or DMSA, respectively. BAL was not evaluated against sodium arsenite in either of these studies. It would be difficult to assume that findings from the studies mentioned above would also be indicative of the findings of similar studies conducted using L or CVAA. The differences in PDH complex inhibition potency between the organic arsenicals and sodium arsenite observed in the present study indicate that the events occurring at the molecular level between As^{+3} and its biochemical target on the PDH complex molecule may be significantly different between sodium arsenite and either L or CVAA.

5.0 CONCLUSIONS AND RECOMMENDATIONS

An in vitro method for screening arsenic antidotes was developed for use at the MREF. This assay uses the inhibition of PDH complex as its basis for quantitation. The assay is effective, inexpensive, and quantitative for comparing the efficacy of reactivators of PDH complex inhibitors which contain As^{+3}. The assay may also be of value in comparing the efficacy of chelating agents for arsenicals containing As^{+5} and other inhibitors of sulfhydryl-containing enzymes, such as mercury, cadmium, and lead.

At present, the screening assay is relatively labor intensive and subject to occasional technical problems that accompany all manual enzyme assay methods. To increase the cost effectiveness of the assay and to improve the overall precision of the results, the assay should be automated. This will also eliminate the need to routinely manipulate solutions that contain XCSM amounts of L, thereby significantly reducing the risk of personal injury and saving personnel time. Plans for the development of an automated procedure for this assay are under way, using the COBAS-FARA centrifugal analyzer which was recently purchased for the MREF.

Finally, there is a need to collect the necessary in vivo data for validation of the results obtained using the in vitro assay. The results obtained using BAL in Task 84-4 (Tissue Distribution of Arsenic in the Rabbit Following Subcutaneous Administration of Lewisite With or Without British Anti-Lewisite Therapy) provide the type of information needed to validate the predictive assay for L. Similar studies need to be conducted with both DMPS and DMSA against L to provide comparative data. Such a comparison can be made a subsection of MREF Task 86-24 (Arsenic Mobilization by DMSA/DMPS Lewisite Exposure).

In the selection of new arsenic antidotes for field use, several factors should be taken into account, one of which being the relative efficacy of the compounds being considered. The results obtained from this study indicate that the PDH complex screening assay does provide a means by which to evaluate the relative efficacy of arsenic antidotes in vitro. Following the necessary in vivo validation studies, it appears that the assay will rapidly and inexpensively provide the U.S. Army with quantitative information

concerning the reactivator/inhibitor molar ratio required to eliminate arsenic toxicity at one of its primary biochemical targets. This information could then be considered along with other quantifiable factors to select the "best available" arsenic antidote for further testing or field use.

6.0 ARCHIVE RECORDS

Records pertaining to the conduct of the study are contained in Battelle Laboratory Record Book Nos. MREF-78 and MREF-89. All original data, as well as the original final report, will be maintained at the MREF until forwarded to USAMRDC at the conclusion of the project or until microfiched and permanently archived at Battelle.

7.0 ACKNOWLEDGMENTS

The names, role in the study, and highest academic degree of the principal contributors in this study, are given in the following list:

Name	Title	Degree
Dr. Ronald L. Joiner	Study Director	Ph.D.
Dr. Ming J. Chang	Scientific Advisor	Ph.D.
Dr. David W. Hobson	Study Supervisor	Ph.D.
Ms. Donna R. DeVore	Technical Supervisor	B.S.
Mr. Thomas H. Snider	Biostatistician	B.S.
Ms. Ramona A. Mayer	Quality Assurance	B.A.

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APPENDIX A

MREF Protocol 16 --- "Pyruvate Dehydrogenase System For Determining the Effectiveness of Arsenic Antidotes"

Pyruvate Dehydrogenase System For Determining the Effectiveness of Arsenic Antidotes

Study performed by Battelle Columbus Laboratories,
505 King Avenue, Columbus, Ohio 43201

1. Study Director: Ronald L. Joiner, Ph.D.

2. Sponsor: U.S. Army Medical Research and Development Command

3. Sponsor Monitor: LTC Howard Johnson, USAMRICD

4. Objective:

To establish an in vitro screening and testing system capable of evaluating the relative effectiveness of candidate antidotal compounds in the prevention and/or reversal of systemic effects resulting from exposure to lewisite and/or other arsenic containing compounds.

5. Experimental Design:

A. General

(1) The overall catalytic activity of the PDH complex is determined spectrophotometrically by monitoring NADH production at 340 nm (25°C). Sodium arsenite, lewisite, chlorovinylarsenous acid, and phenyldichlorarsine are evaluated individually in the PDH assay system with regard to the minimal concentration and time required to achieve 100 percent inhibition of catalytic activity.

(2) Once the appropriate concentration of each inhibitor has been established in the PDH assay system, candidate antidotal agents (such as BAL, DMP and DMSA) are evaluated for their ability to reverse and/or prevent the effect of arsenic on PDH activity. Potential antidotal agents are evaluated with regard to the effective concentration and time required to achieve restoration of intact PDH activity.

(3) In the unlikely event that commercial sources of the highly purified PDH complex are not available, the enzyme complex can be purified at the MREF by harvesting a sufficient number of mouse kidneys at one time so as to provide a homogenous enzyme preparation for use during the entire Task (the enzyme complex can be frozen without appreciable loss of activity).

B. Standard Assay System

(1) The standard assay mixture is composed of 0.2 umoles thiamine pyrophosphate, 1.0 µmoles $MgCl_2$, 2.5 µmoles NAD^+, 0.13 µmoles of CoA, 2.6 µmoles cysteine hydrochloride, 2.0 µmoles potassium pyruvate, and 50 µl of 0.02 M potassium phosphate buffer, pH 8.0.

(2) The reaction is initiated by addition of a fixed amount of PDH complex formulated in a volume so as to make the final volume of the assay mixture 1.0 ml. The addition of the enzyme system as the final step facilitates controlled exposure of the PDH complex to an inhibitor or antidotal agent prior to mixing with the substrate at time zero.

C. Measurement of NADH

(1) Assays of PDH complex activity are made in matched quartz cuvettes at 340 nm by monitoring the increase in absorbance due to NADH production.

(2) The potential conversion of NAD^+ to NADH by sources other than the PDH complex must be determined for each experimental phase and, where necessary, appropriate corrections made in the kinetic data for PDH activity.

6. Decontamination:

A. All non-disposable glassware is decontaminated with 5% sodium hypochlorite prior to removal from the toxic fume hood.

B. The contents of cuvettes are poured into a 4-liter plastic beaker containing approximately 2000 ml of 5% sodium hypochlorite. The cuvettes are washed with 5% sodium hypochlorite prior to total immersion into the 4-liter beaker.

C. All decontamination solutions and decontaminated wastes are collected for proof of decontamination prior to being drummed and disposed of in a hazardous land fill.

D. No arsenic-containing waste can be incinerated or released to the environment in discharge water.

7. Records to be Maintained:

A. CSM accountability log and inventory

B. Dosage preparations and administrations

C. Preparation of reagents

D. In vitro system operation parameters and test conditions

E. Experimental data

F. Statistical methodology used

G. Results of decontamination monitoring

8. Reports:

A final report is prepared and submitted within 30 days after completion of the Task. The final report format must comply with the USAMRDC SOP, "Procedures for Preparing and Processing Medical Research and Development Contractor and Grantee Reports."

9. Approval Signatures:

Ronald L. Joiner, Ph.D.
Study Director

October 16, 1984
Date

LTC Howard Johnson
USAMRDC Monitor

17 Oct 84
Date

10. Amendment A - October 14, 1985:

This is to document some errors in MREF Protocol 16 (Pyruvate Dehydrogenase System For Determining the Effectiveness of Arsenic Antidotes) and state their corrections. This amendment also clarifies procedural information regarding the preparation of sample component solutions.

1. Page 2, Section 5.B.(1)

Amounts of $MgCl_2$ and cysteine hydrochloride are 0.5 µmoles and 2.08 µmoles, respectively.

There is no potassium pyruvate used in this protocol. Sodium pyruvate is used.

50 µl of 0.02 M potassium phosphate buffer, pH 8.0, is replaced with TRIS (tromethamine) buffer, which experimentally was found to yield better results.

The following information clarifies the procedure used to make an analytical sample:

	Stock Conc.(mM)	Mol. Wt.	Stock Conc. (wt/Vol.H_2O)	Component in Standard Assay Sample (µmoles)	Vol. Used µl/Sample
TPP	4	460.8	90.5/49.1 ml	0.2	50
NAD^+	25	663.4	98.5/5.94 ml	2.5	100
CoA	2.6	767.5	9.4/4.72 ml	0.13	50
Cys HCl	26	157.6	203.4/49.6 ml	2.08	80
Na pyruvate	20	110.0	109.1/49.6 ml	2.0	100
EDTA	20	416.2	412.9/49.6 ml	0.5	25
$MgCl_2$	20	203.3	222.0/54.6 ml	0.5	25
$CaCl_2$	20	147.02	200.5/68.2 ml	0.5	25
TRIS	0.2	157.6	1.577 g/50 ml	90.0	450
PDH Enzyme	Neat as supplied				50
				Subtotal	955 µl

955 µl + 45 µl of inhibitor = 1000 µl

Total Sample Volume = 1.0 ml

11. Approval Signatures for Amendment A:

Ronald L. Joiner, Ph.D.
Study Director

16 OCT 85
Date

I.TC(P) Howard C. Johnson
USAMRDC Monitor

21 Oct 85
Date

12. Amendment B - October 8, 1986:

The following amendments to MREF Protocol 16 (Pyruvate Dehydrogenase System for Determining the Effectiveness of Arsenic Antidotes) have been found necessary in order to examine the effectiveness of antidotes using XCSM concentrations of Lewisite. These changes increase the number of samples that can be performed, decrease the technical time required per sample, and decrease the amount of PDH enzyme required per sample.

A. Page 1, Section 5.A.(1)

NADH production at 340 nm will be monitored at 30 C instead of 25 C.

The working minimal concentration and time target of 100 percent inhibition for arsenite, Lewisite, chlorovinylarsenous acid, and phenyldichlorarsine will be changed to 90 percent inhibition to allow for experimental variability in PDH and the inhibitory action of the arsenical compounds.

B. Page 1, Section 5.A.(2)

Because of buffer solubility and possible antidotal efficacy limitations, candiate antidotes will be evaluated relative to the minimal molar ratio (antidote/inhibitor) and time required for maximal restoration of PDH activity within the practical limits of solubility of each antidote.

C. Page 2, Section 5.C.(1)

Assays of PDH complex activity will be made using disposable, plastic cuvettes that are manufactured to be optically clear, specifically at 340 nm. Besides eliminating the time required for washing optically-matched quartz cuvettes, this also eliminates the potential for inhibition of PDH activity from glass-retained impurities.

D. Page 4, Section 10

The weight of all stock components per ml of water is in milligram amounts.

Sodium pyruvate concentration has been changed to 10 mM, and the weight per volume of water (w/v) ratio is 54.55 mg/49.6 ml. The amount of sodium pyruvate in a standard assay sample is now 1.0 µM.

PDH enzyme is now diluted as follows: 0.1 ml stock enzyme + 1.4 ml TRIS buffer.

Total sample volume will consist of 955 µl of sample + 45 µl of inhibitor + 45 µl of antidote = 1,045 µl or 1.045 ml.

13. Approval Signatures for Amendment B:

Ronald L. Joiner, Ph.D.
Study Director

October 27, 1986
Date

MAJ(P) J. Bruce Johnson
USAMRDC COR

28 October 1986
Date

APPENDIX B

MREF SOP-83-7 --- "Enzyme Inhibition by Exempt G and V Agents, L and Other Arsenic Compounds, and Mustard"

STANDARD OPERATING PROCEDURE
MREF SOP-83-7

TITLE: Enzyme Inhibition by Exempt G and V Agents, L and Other Arsenic Compounds, and Mustard

LABORATORY: MREF SOP Approval Date: Revised August 15, 1984

EXPIRATION DATE: 12/1/86

PLACE OF OPERATION OR TEST: Room 4 or Room 17

This standard operating procedure (SOP) has been prepared as prescribed by Contract DAMD17-83-C-3129 and will be effective for one year from date of approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been trained properly and instructed in its provisions and attest to this requirement by causing them to affix their signatures on page 2.

A copy of this SOP will be posted at the job site at all times.

Approved By: Donald W Cagle 12/2/85
Signature/Date

Donald W. Cagle, CIH, Safety/Security Officer
Printed Name/Title

STANDARD OPERATING PROCEDURE
MREF SOP-83-7

TITLE: Enzyme Inhibition by Exempt G and V Agents, L and Other Arsenic Compounds, and Mustard

LABORATORY: MREF SOP Approval Date: Revised August 15, 1984

PLACE OF OPERATION OR TEST: Room 4 or Room 17

This standard operating procedure (SOP) has been prepared as prescribed by Contract DAMD17-83-C-3129 and will be effective for one year from date of approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been trained properly and instructed in its provisions and attest to this requirement by causing them to affix their signatures on page 2.

A copy of this SOP will be posted at the job site at all times.

Submitted By: Werner L. Margard 12-4-84
Signature/Date

Werner L. Margard, Assistant Manager
Printed Name/Title

Recommending Approval: Ronald L Joiner 12/4/84
Signature/Date

Ronald L. Joiner, Ph.D., Manager
Printed Name/Title

Approved: Donald W. Cagle 12/4/87
Signature/Date

Donald W. Cagle, CIH, Safety/Security Officer
Printed Name/Title

SIGNATURES

I have read and understand the contents of MREF SOP-83-7.

Signature	Date	Signature	Date
Pamela H. Kinney			
David M. Wilson	4 SEP 86		
Donna R. DeVore	9/15/86		
Linda K. Adams	9-15-86		

STANDARD OPERATING PROCEDURE 83-7

Enzyme Inhibition by Exempt G and V Agents,
L and Other Arsenic Compounds, and Mustard

A. Statement of Work: Exempt Chemical Surety Materiel (XCSM; dilute, less than surety) used for determination of enzyme inhibition will be received by an MREF staff member from the appropriate MREF Custodian. These solutions will be further diluted in Room 4 or 17 to establish an appropriate level of inhibition from the enzymes being tested by a spectrophotometric procedure.

B. Responsibility:

1. Personnel Qualifications. Technical staff will consist of at least one individual designated by the Manager of the Medical Research and Evaluation Facility (MREF) as authorized to receive exempt (diluted) agents from Room 02B of the MREF. Technical staff must have a Chemical Personnel Reliability Program (CPRP) approval and must be current with the requirements of the MREF Facility Safety and Surety Plan and all applicable MREF standard operation procedures.

2. Leaders. Leaders of each operation in Room 4 or Room 17 of the MREF will be designated by the Custodian issuing exempt (dilute) agents for that operation. That Custodian will insure that the following are observed:

a. That agents are issued exclusively to personnel who have been designated in writing from the Manager, MREF, as authorized to receive CSM.

b. Maintain control and accountability of agent.

c. Adequate supply of approved protective equipment is available at all times to personnel at their work site.

d. All leader and technical staff responsibilities specified in MREF Facility Safety and Surety Plan.

e. That each employee has been trained in the techniques of administering first aid and self aid.

f. Work under this SOP will be performed only in the room(s) designated.

g. That no food, beverage, or tobacco will be consumed or brought into the laboratory.

h. That the safety requirements of this SOP, as well as normal laboratory safety, are maintained.

i. That decontamination solutions are present prior to handling agents.

j. That only proper quantities of dilute agents leave the room and that they are properly contained and labeled.

k. That the SOP is read/signed by all technical staff using agents.

3. Technical Staff will be responsible for abiding by requirements set forth in paragraph B2. In addition, they must:

a. Use personal protective equipment provided. Develop safe work habits by following good laboratory practice to protect themselves and fellow workers from injury and to prevent damage to material, equipment, and facilities.

b. Not perform surety agent operations without the presence of a qualified second person with visual contact (Buddy System).

4. Additional supervision and guidance will be provided by other appropriate individuals, which include the following:

a. Biological Sciences Department Staff

(1) Health and Safety

(2) Radiological Safety

(3) Veterinary

(4) Pathology

(5) Toxicology

b. Chemistry Department Staff

(1) Analytical

(2) Quality Control

c. Laboratory-wide Staff

(1) Health and Safety

(2) Radiological Safety

(3) Environmental Control

5. The organization involved in this research is the Medical Research and Evaluation Facility of Battelle Memorial Institute's Columbus Division, 505 King Avenue, Columbus, Ohio 43201.

C. Materials to be Used:

Anticholinesterase agents:

GA
GB
GD
VX

Vesicant agents:

Lewisite
Chlorovinylarsenous acid
Phenyldichloroarsine
Sodium arsenite

D. Tools and Equipment to be Used:

Safety equipped cart, hood, freezer (locked), refrigerator (locked), latex gloves, labels, 10-ml volumetrics, pipets, support block, decontaminant solution (5% NaOH for G and V agents; 5% sodium hypochlorite for arsenic compounds and mustard), first aid kit, absorbent paper, kraft paper, 4-liter beaker, squirt bottles, wiping tissues, isopropanol , methanol.

E. Hazards Involved:

1. GA, GB, GD, and VX are nerve agents. The principal hazard from these agents (particularly GB) is vapor inhalation with consequent absorption through the respiratory tract. All of the agents may be absorbed on contact through the intact skin, through eyes, and through gastrointestinal tract if ingested. All are highly toxic and quick acting.

2. VX and GD are persistent and are primarily a liquid hazard. Inadvertent skin contact is a lethal hazard from these agents. Percutaneous exposure to either the liquid or vapor may be fatal. The toxicity by the percutaneous route is much higher than by the respiratory route.

3. Lewisite

   a. Lewisite (L) is a vesicant, and acts on the eyes, the lungs, and the skin. It burns and blisters the skin or any other part

of the body it comes in contact with. It damages the respiratory tract when inhaled and causes vomiting and diarrhea when absorbed.

b. Lewisite causes immediate pain upon exposure.

c. Lewisite is much more dangerous as a liquid than as a vapor. The liquid will cause severe burns of the eyes and skin, while field concentrations of vapors are unlikely to cause significant injury.

d. Lewisite is a volitile liquid that hydrolyzes readily in humid air. It can be absorbed through the skin in sufficient quantities to cause systemic arsenic poisoning before vesication can appear.

4. Chlorovinylarsenous acid

Chlorovinylarsenous acid is the relatively stable product of lewisite hydrolysis. It is a strong acid and, as such, constitutes the principal means of irritation from lewisite exposure. The symptoms listed above for lewisite are probably more due to chlorovinylarsenous acid than its precursor.

5. Phenyldichloroarsine (CAS 696-28-6)

a. Phenyldichloroarsine is highly toxic by inhalation, ingestion, and skin absorption.

b. It is a severe irritant to skin, eyes, and other tissues as characterized by dermatitis, blistering, conjunctivitis, and tracheal irritation. It also casues nausea and vomiting, diarrhea, and jaundice.

6. Sodium arsenite

a. Inorganic trivalent arsenic compounds are corrosive to the skin. Prolonged contact results in hyperemia and vesicular or pustular eruption. The moist mucous membranes are most sensitive to the irritant action.

b. Acute systemic effects following ingestion include throat constriction, difficulty in swallowing, burning epigastric pain, vomiting, watery diarrhea, and muscle spasms.

c. Acute arsenical poisoning due to inhalation is characterized by cough, chest pain, dyspnea, vertigo, headache, and general malaise and fatigue, followed by gastro-intestinal disturbances.

d. Of particular interest is the fact that arsenic and inorganic arsenic compounds have been declared suspected human carcinogins (29 CFR 1910) and therefore must be handled in accordance to strict standards for the use of these substances.

7. Mustard

a. The persistence of hazard from mustard vapor or liquid depends on the extent of contamination by the agent.

b. Of particular importance is the fact that mustard (HD) has been declared to be a known carcinogen and therefore must be handled in accordance to strict standards for the use of these substances.

c. HD is a liquid of relatively low volatility. It is a powerful blister agent that burns the skin, eyes and lung tissues. Exposure to even a slight concentration of HD is capable of causing severe burns that appear 4 to 25 hours after exposure.

F. Safety Requirements:

1. Hoods.

Hood face velocity must average 100 ±15 lfpm for exempt chemical surety materiels. This average is computed from individual readings taken in approximately each square foot of hood face (usually nine readings). If these readings are met, smoke tubes will be used monthly to ensure no unacceptable turbulent flow situations exist. A hood alarm that indirectly senses average face velocity provides a reasonably reliable indication of proper air flow. Due to variations that may occur, checks of the hood flow are made with a velometer, vaneometer or other similar device. No XCSM or agent-contaminated equipment will be within 20 cm of the face of the hood.

2. Protective Equipment.

When working with exempt agents, the following clothing and protective gear is required as a minimum:

Scrub suit
Laboratory coat
Safety shoes
Safety glasses
Shoe covers
2 pairs of latex gloves

All provisions of the MREF Facility Safety and Surety Plan apply to the checking and testing of gloves, respirators, and other protective equipment.

3. Respirators.

An air-supplied respirator is assigned to each individual and is present in the room where each individual is working. Daily observations will be made on the respirators. If the respirators are used, they must be washed thoroughly with soap and water.

4. First Aid.

A first-aid kit containing 5% sodium hypochlorite, 10% sodium hydroxide in ethanol, and water in squirt bottles (these bottles will be labelled, dated and the contents changed every month), three atropine/2-PAM auto-injectors per person with currently acceptable lot numbers, an ampule of BAL in oil, and gauze pads will be located in the room. The location of the nearest eye-wash fountain, shower, and fire extinguisher will be known to all workers before work begins. First-aid instructions will be stored with the first-aid kit.

G. Procedures:

Step	Description	Specific Instructions
1 Receipt of XCSM	The XCSM solution is received by an individual designated by the MREF Manager from the storage hood or freezer in Room 2 or the freezer in Room 17. The XCSM will be double contained for transport.	The individual is wearing a scrub suit, laboratory coat, and 2 pairs of latex gloves. The transfer cart is equipped with a spill tray, a 4-liter bucket containing approximately 2 liters of the decontaminant, absorbant paper, and vermiculite.

Step	Description	Specific Instructions
2 Transfer	The XCSM solution is transported to room 4 or 17, removed from the secondary container, and placed in a support block in the hood.	The hood is lined with absorbant paper and contains a 4-liter bucket with approximately 2 liters of decontaminant, a squeeze bottle of decontaminant, and absorbant paper.

Step	Description	Specific Instructions
3 Further Dilution	Dilutions of the XCSM solution are made in an approved hood. The diluent is added to the dilution container before uncapping the XCSM solution.	A scrub suit, laboratory coat, and two pairs of latex gloves are worn. Quantities of the stock solution are transferred using disposable pipettes. No mouth pipetting is permitted. All dilution operations are conducted over absorbant paper. Containers are capped as soon as practical after making the dilution. Contaminated pipettes are placed directly into the decontamination bucket. After the dilutions are made, the outer latex gloves are removed and placed in the decontamination bucket.

Step	Description	Specific Instructions
4 Preparation for Experiment	The containers with the working concentrations are placed in a hood in Room 4 or Room 17. All other solutions necessary for the experiment (e.g. pipettes, vials, cuvettes) should already be in the hood.	A cart is used to move the working solutions. The hood is lined with absorbant paper and contains a 4-liter bucket with approximately 2 liters of decontaminant and a squeeze bottle of decontaminant. The vials and cuvettes will be in a rack or container to prevent tipping.

Step	Description	Specific Instructions
5 Dosing and Analysis	The working solution of XCSM is mechanically pipetted into the vial as required. A separate pipette is used to deliver the required "dose" to the cuvette. The cuvette is capped with the cuvette cover or with Parafilm, mixed by inverting several times, removed from the hood, and placed in the cell holder in the spectrophotometer.	Double latex gloves are worn during all XCSM operations. The spectrophotometer is located on the laboratory bench immediately adjacent to the hood in Room 4 or in Room 17. After mixing, the capped cuvette is removed from the hood and placed in the spectrophotometer. The outer latex gloves are then removed and placed in the decontamination bucket. After the analysis, the cuvette is removed from the spectrophotometer (while wearing 2 pairs of latex gloves), uncapped, and the contents gently emptied into the decontamination bucket in the hood. Then the cuvette is placed in the decontamination bucket in the hood, ensuring that the decontamination solution fills the cuvette. The cap/parafilm cover is placed into the decontamination bucket and submerged.

Step	Description	Specific Instructions
6 Decon-tamination of Unused Agent	If the amount of XCSM is not in sufficient quantity to warrant saving for operations on a successive day, it is decontaminated.	To decontaminate the XCSM in the container, gently pour any remaining XCSM into the decontamination bucket. Rinse the container three times with the decontaminant using the squeeze bottle and then totally submerge the container in the bucket.

Step	Description	Specific Instructions
7 Decontamination and Clean-Up	All contaminated glassware in hoods is submerged in decontamination solution overnight. The next morning the glassware is removed from the decontamination solution, drained, and rinsed carefully with fresh decontamination solution before being removed from the hood for normal washing. All solid and liquid waste material remain in the decontamination bucket in the hood overnight. The following day, the liquid is adsorbed onto paper or similar absorbing material with the solid waste; all wastes are then placed into a plastic disposal bag, taped closed, and held until incinerated.	All exempt-level protective equipment is worn during decontamination.

Step	Description	Specific Instructions
8 Spill Procedure	If the XCSM is spilled within the hood, decontaminant is applied from the supply in the hood. Forceps are used to dip absorbent paper into the decontaminant solution. If the exempt agent is spilled outside the hood, the person cleaning up the spill first dons his/her respirator before following the same procedure to decontaminate as for in-hood spills.	Exempt CSM spills inside or outside of the hoods are handled in similar fashion. The decontamination solution located within the hood is gently poured or swabbed with soaked absorbent paper held with forceps on the area in an amount that is at least tenfold in excess of the spill. The decontaminant/CSM mixture is then absorbed with Fuller's Earth or other absorbant and deposited into plastic bags. This cleaning/absorption procedure is repeated again. The bag is then sealed and held until incinerated.

H. Emergency Procedures: Toxic agent spills, decontaminations, personnel actions, precautions, etc., for unplanned incidents will be handled according to procedures in MREF-SOP-24: CSM Spills and Safety.

I. Emergency First-Aid Procedures: As follows and as stated in MREF-SOP-18: Response to Emergency Situations.

1. Make sure that you protect yourself from contamination by the casualty. Mask if in doubt.

2. Push Panic Button

3. Personnel exposed to a toxic agent will be removed immediately to a shower area where washing and first aid can be administered by co-workers. If there is any question about the source of contamination, place the victim under the emergency shower, turn on the shower, and remove the wet clothing while still in the shower. Wash the victim down with soap; do not scrub as this may enhance penetration.

4. Emergency treatment for G and V agents, L and other arsenic compounds:

   a. Decontamination when the source of contact is certain.

      (1) Skin: Wash skin at once with diluted chlorine-type bleach and rinse with copious amounts of water.

      (2) Eyes: Use water only; rinse a minimum of ten minutes at the eyewash fountain.

   b. Transfer the victim to clean area and thoroughly decontaminate with 5% sodium hypochlorite only in the areas below the eyes in the position in which the victim is being held. Decontaminate with dry tissue or absorbent paper followed by water in areas close to the eyes.

   c. Place victim in shower and remove clothing.

   d. If victim has symptoms of anticholinesterase poisoning beyond miosis, inject him with the contents of the atropine/2-PAM autoinjector at intervals of 5-10 minutes up to a maximum of three injections. Note time of each injection on the victim for reference by physician.

   e. If victim has stopped breathing, employ resuscitation with the ambu-bag immediately. Use the atropine autoinjector after you have successfully succeeded in restoring respiration.

5. The decontaminated individual will be transported by ambulance to University Hospital.

6. In the event of any exposure, the MREF Manager (879-5118) should be summoned after the emergency is taken care of.

   The following individuals must be contacted by the MREF Manager or designee:

   a. Manager, Biological Sciences Department - 424-7065

   b. Manager, Health Services - 424-6337

   c. MREF Safety and Security Officer - 424-7622

   d. Chief, USAMRDC Chemical Surety/Safety Office - (301)-671-4433

APPENDIX C

Tables

TABLE 3.1. DATA OBTAINED FROM INHIBITION OF PDH COMPLEX ACTIVITY USING VARIOUS CONCENTRATIONS (mM) OF SODIUM ARSENITE ($NaAsO_2$). PERCENT PDH COMPLEX ACTIVITY IS EXPRESSED RELATIVE TO CONTROL SAMPLES USED TO MONITOR THE ACTIVITY OF SEQUENTIAL PREPARATIONS OF PDH COMPLEX. THE CONTROLS ALSO REPRESENT A SODIUM ARSENITE SAMPLE CONCENTRATION OF ZERO.

Sample Name	Inhibitor mM	PDH U/ℓ	Absorbance dA/min.	PDH (%)	Regress. Coeff.(r)
Control #1	0	154.9	0.0461	100	0.99
Control #2	0	115.4	0.0343	100	0.99
Control #4	0	137.1	0.0408	100	0.99
Control #5	0	128.6	0.0383	100	0.99
$NaAsO_2$	0.1	141.6	0.0421	103.3	1
$NaAsO_2$	0.1	126.2	0.0376	109.4	0.99
$NaAsO_2$	0.1	136.4	0.0406	88.1	1
$NaAsO_2$	0.5	84.6	0.0252	54.6	1
$NaAsO_2$	0.5	75.5	0.0225	65.4	1
$NaAsO_2$	0.5	85.4	0.0254	62.3	1
$NaAsO_2$	0.5	86.1	0.0256	62.8	0.98
$NaAsO_2$	1	55.4	0.0165	35.8	1
$NaAsO_2$	1	63.3	0.0188	49.2	1
$NaAsO_2$	1.5	46.2	0.0137	35.9	0.99
$NaAsO_2$	1.5	39.8	0.0118	25.7	1
$NaAsO_2$	2	30.6	0.0091	23.8	0.99
$NaAsO_2$	2	38.0	0.0113	27.7	1
$NaAsO_2$	2.5	38.0	0.0113	27.7	1
$NaAsO_2$	2.5	33.2	0.0099	28.8	1
$NaAsO_2$	2.5	63.3	0.0188	49.2	1
$NaAsO_2$	3	29.4	0.0088	22.9	1
$NaAsO_2$	3	24.6	0.0073	18	1
$NaAsO_2$	5	30.1	0.0089	21.9	1
$NaAsO_2$	5	16.2	0.0048	14.1	1
$NaAsO_2$	5	16.9	0.005	13.2	0.99
$NaAsO_2$	10	9.1	0.0027	7.9	0.93
$NaAsO_2$	10	11.2	0.0033	8.7	0.99
$NaAsO_2$	10	11.0	0.0033	9.5	0.99

TABLE 3.2. DATA OBTAINED FROM THE INHIBITION OF PDH COMPLEX ACTIVITY USING VARIOUS CONCENTRATIONS (mM) OF LEWISITE. PERCENT PDH COMPLEX ACTIVITY IS EXPRESSED RELATIVE TO CONTROL SAMPLES USED TO MONITOR THE ACTIVITY OF SEQUENTIAL PREPARATIONS OF PDH COMPLEX. THE CONTROLS ALSO REPRESENT A LEWISITE SAMPLE CONCENTRATION OF ZERO.

Sample Name	Inhibitor mM	PDH U/ℓ	Absorbance dA/min.	PDH (%)	Regress. Coeff.(r)
Control #1	0	223.9	0.0666	100	1
Control #2	0	188.2	0.056	100	1
Control #3	0	218.4	0.065	100	1
Control #4	0	138.9	0.0413	100	1
Control #5	0	215.4	0.0641	100	1
Control #6	0	131.5	0.0391	100	1
Control #7	0	222.8	0.0663	100	1
L	0.000397	119.9	0.0357	86.3	1
L	0.000794	119.8	0.0356	86.2	1
L	0.00158	108.5	0.0323	78.2	1
L	0.003125	180.8	0.0538	83.9	1
L	0.00625	153.2	0.0456	71.1	1
L	0.0125	113.4	0.0337	52.6	1
L	0.025	62.2	0.0185	28.9	1
L	0.05	40.3	0.012	21.4	1
L	0.1	13.0	0.0039	6.9	1
L	0.2	6.8	0.002	3.6	0.99
L	0.313	1.2	0.0004	0.9	0.28
L	0.625	4.3	0.0013	3.2	0.85
L	1.25	-2.7	-0.0008	-2.1	-0.48
L	2.5	-1.6	-0.0005	-0.7	-0.86
L	5	-2.6	-0.0008	-1.2	-0.87
L	10	-0.6	-0.0002	-0.3	-0.21
L	20	-8.5	-0.0025	-3.8	-0.75

TABLE 3.3. DATA OBTAINED FROM INHIBITION OF PDH COMPLEX ACTIVITY USING VARIOUS CONCENTRATIONS (mM) OF CVAA. PERCENT PDH COMPLEX ACTIVITY IS EXPRESSED RELATIVE TO CONTROLS USED TO MONITOR THE ACTIVITY OF SEQUENTIAL PREPARATIONS OF PDH COMPLEX. THE CONTROLS ALSO REPRESENT A CVAA SAMPLE CONCENTRATION OF ZERO.

Sample Name	Inhibitor mM	PDH U/ℓ	Absorbance da/min.	PDH (%)	Regress. Coeff.(r)
Control #1	0	216.3	0.0644	100	0.99
Control #2	0	223.9	0.0666	100	0.99
Control #3	0	204.0	0.0607	100	0.99
CVAA	0.003125	179.6	0.0534	88	1
CVAA	0.003125	183.9	0.0547	82.1	1
CVAA	0.00625	130.0	0.0387	63.7	1
CVAA	0.00625	131.6	0.0392	60.9	1
CVAA	0.0125	77.8	0.0232	34.8	1
CVAA	0.0125	83.8	0.0249	38.7	1
CVAA	0.025	50.3	0.015	23.2	1
CVAA	0.025	38.2	0.0114	17.1	1
CVAA	0.05	14.4	0.0043	6.7	0.92
CVAA	0.05	18.9	0.0056	8.4	1
CVAA	0.1	9.2	0.0028	4.1	0.99
CVAA	0.1	9.3	0.0028	4.3	0.99
CVAA	0.2	4.8	0.0014	2.1	0.99
CVAA	0.2	4.0	0.0012	1.9	0.94
CVAA	0.4	0.4	0.0001	0.2	0.19
CVAA	0.4	-0.2	0.0000	-0.1	-0.15

APPENDIX D

Figures

FIGURE 2.1. PDH COMPLEX ACTIVITY

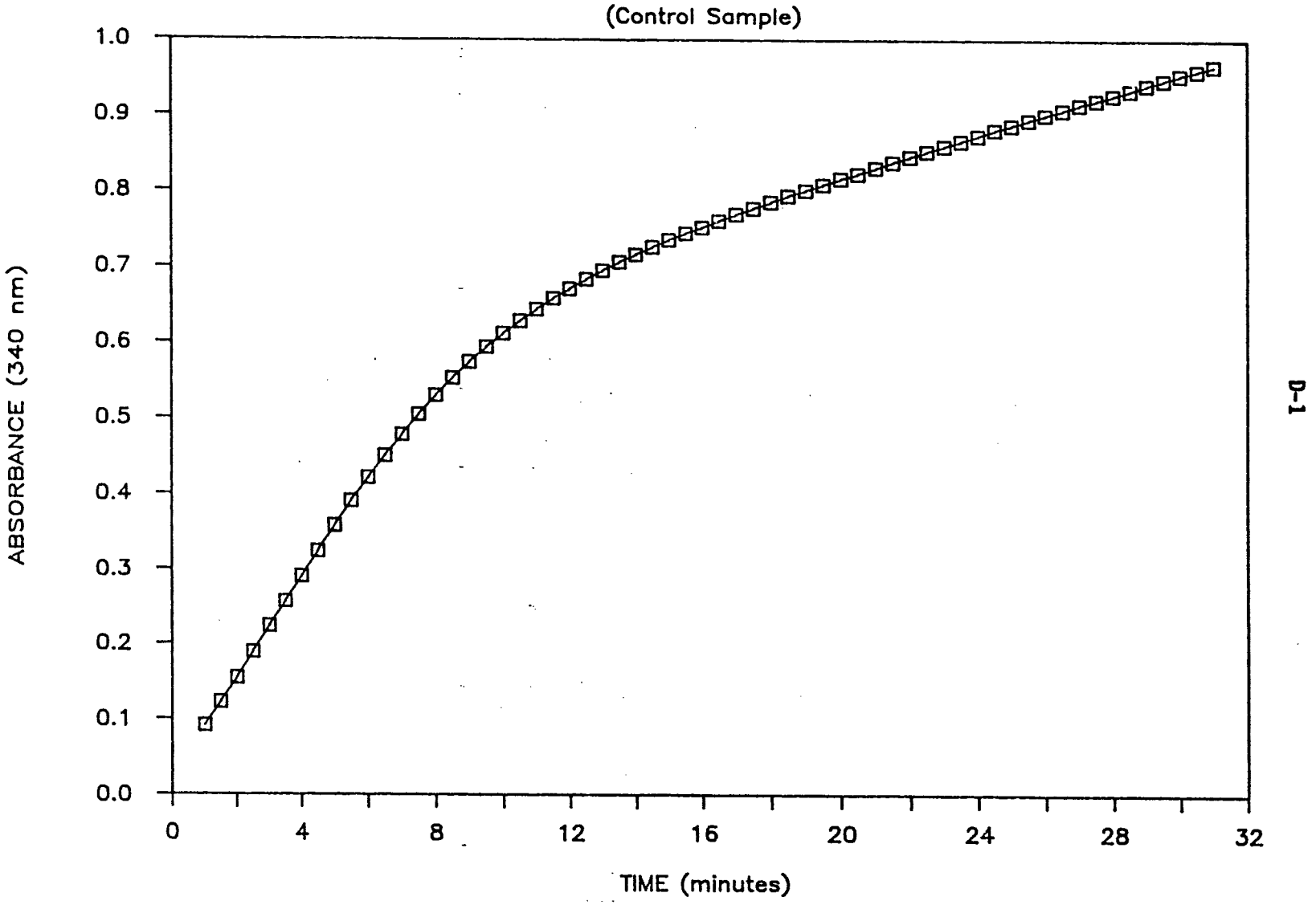


FIGURE 3.1. OBSERVED INHIBITION OF PYRUVATE DEHYDROGENASE

BY SODIUM ARSENITE (+) WITH INVERSE EXPONENTIAL REGRESSION CURVE (___)
AND 95% CONFIDENCE LIMITS (---) IN DEIONIZED WATER

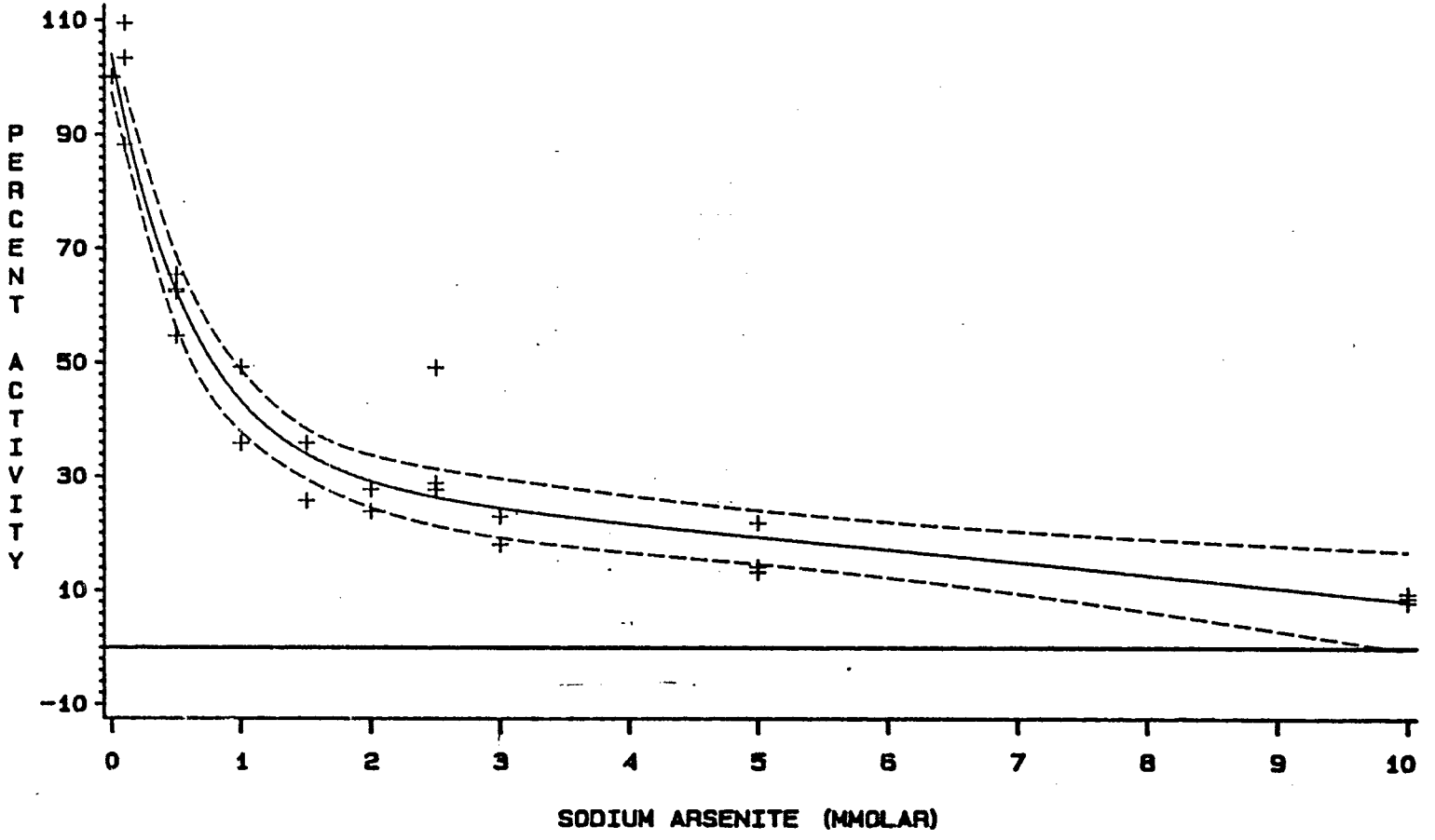


FIGURE 3.2. OBSERVED INHIBITION OF PYRUVATE DEHYDROGENASE
BY LEWISITE (+) WITH INVERSE EXPONENTIAL REGRESSION CURVE (___)
AND 95% CONFIDENCE LIMITS (---) IN DEIONIZED WATER

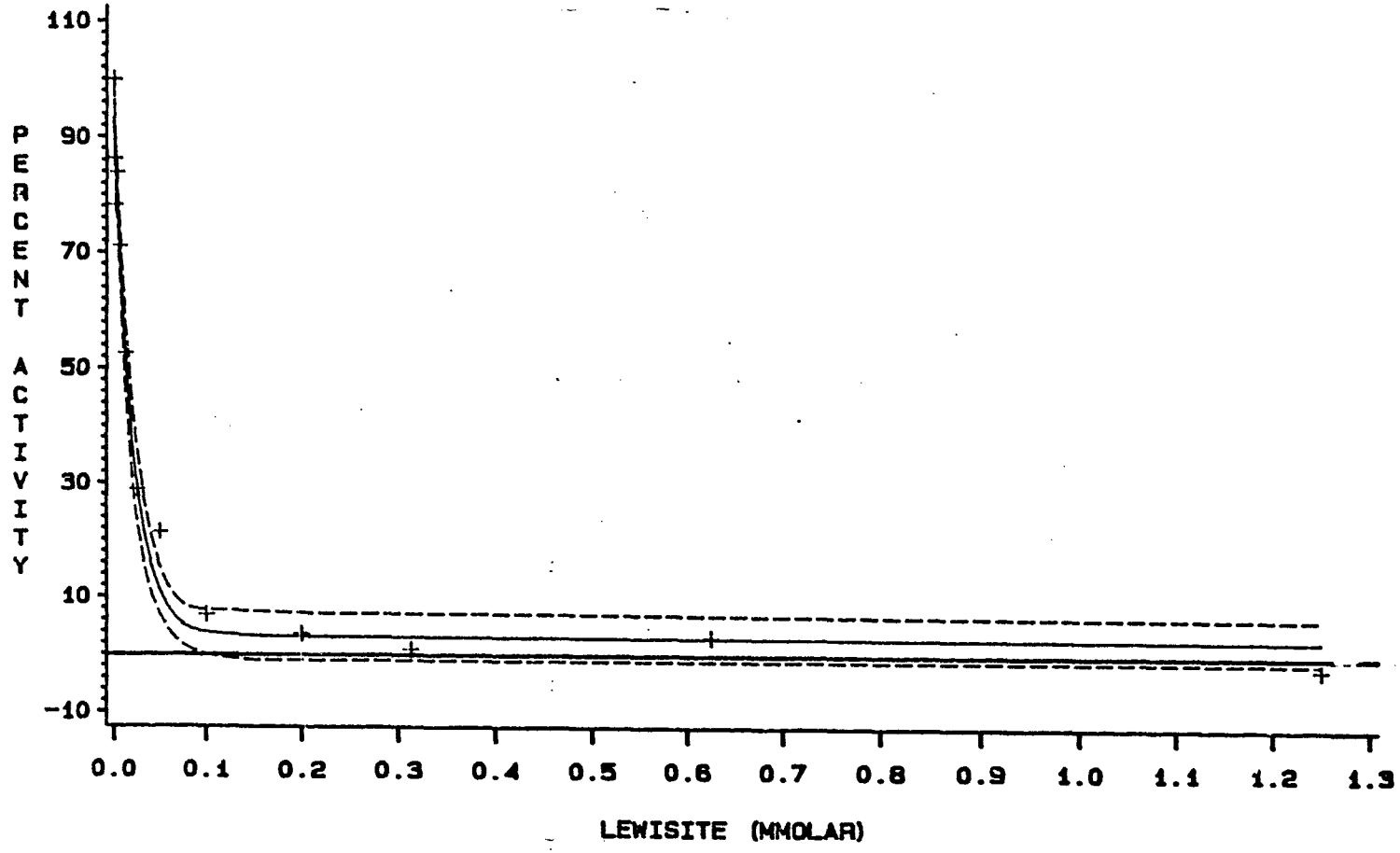


FIGURE 3.3. OBSERVED INHIBITION OF PYRUVATE DEHYDROGENASE

BY CHLOROVINYLARSENOUS ACID (+) WITH INVERSE EXPONENTIAL REGRESSION CURVE (___)
AND 95% CONFIDENCE LIMITS (---) IN DEIONIZED WATER

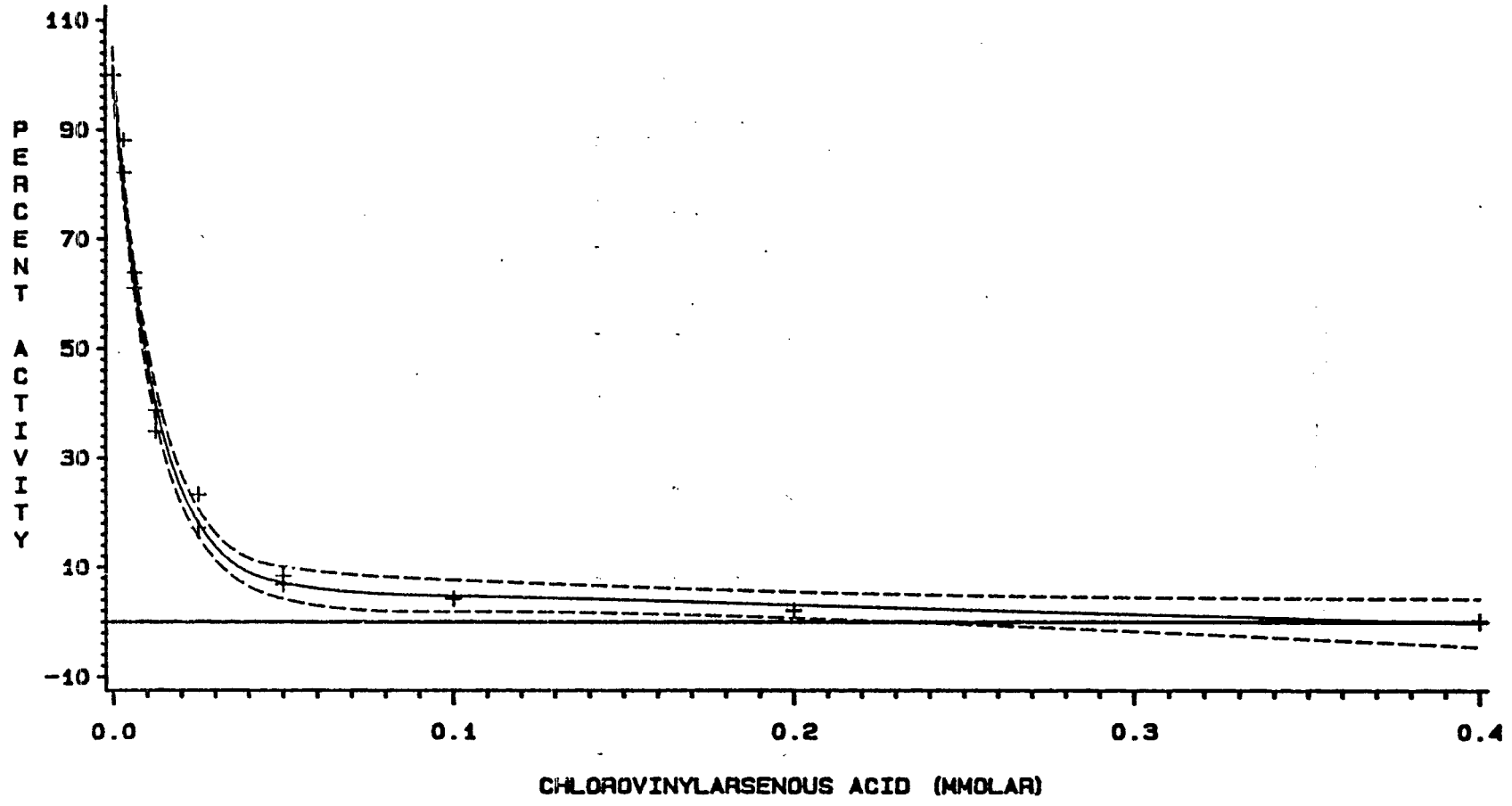


FIGURE 3.4. INHIBITION OF PYRUVATE DEHYDROGENASE
BY SODIUM ARSENITE, LEWISITE,
AND CHLOROVINYLARSENOUS ACID IN DEIONIZED WATER

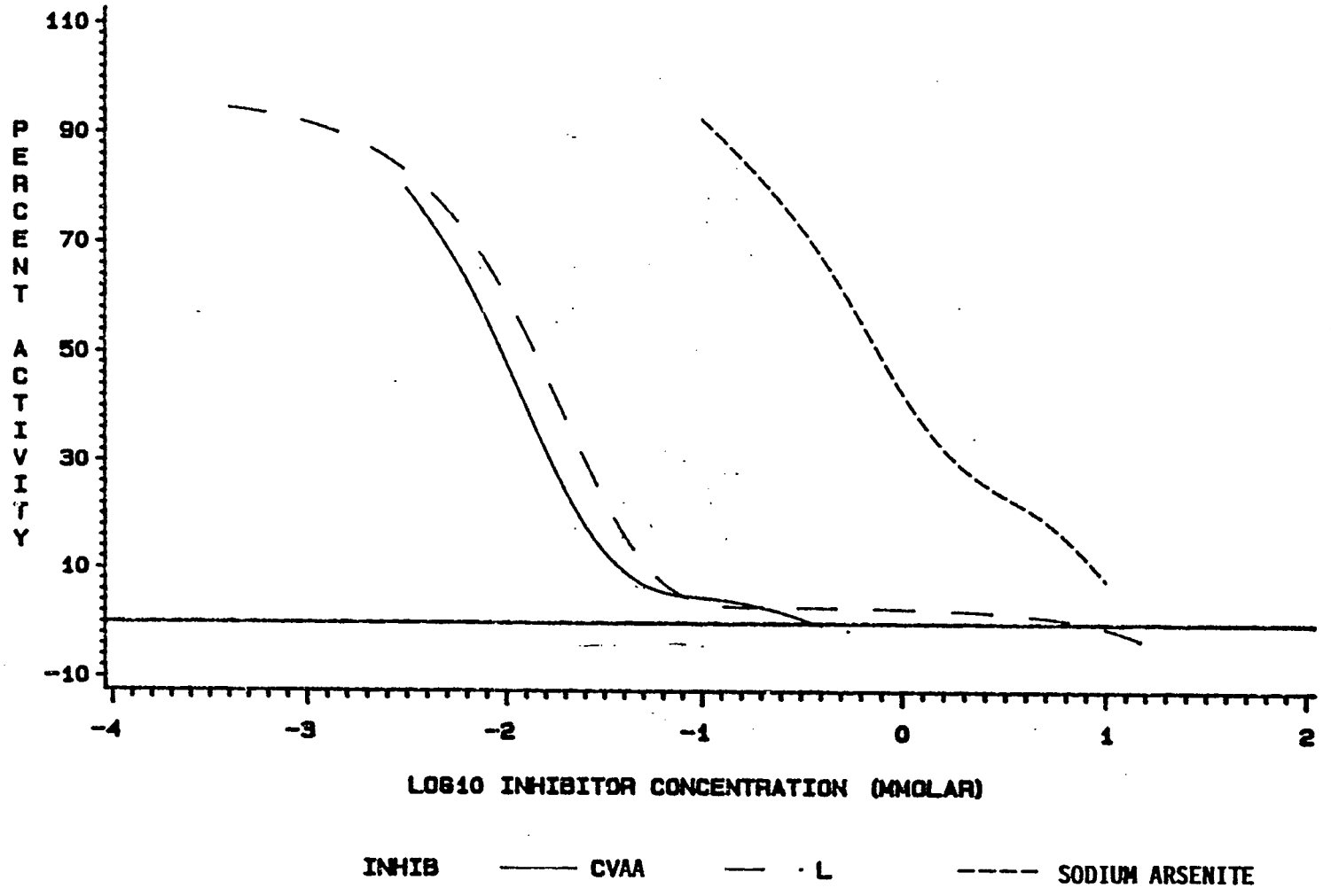


FIGURE 3.5.1. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY SODIUM ARSENITE USING BAL (X)
WITH ONE STANDARD DEVIATION (-) SHOWN ABOUT THE MEAN AT EACH RATIO

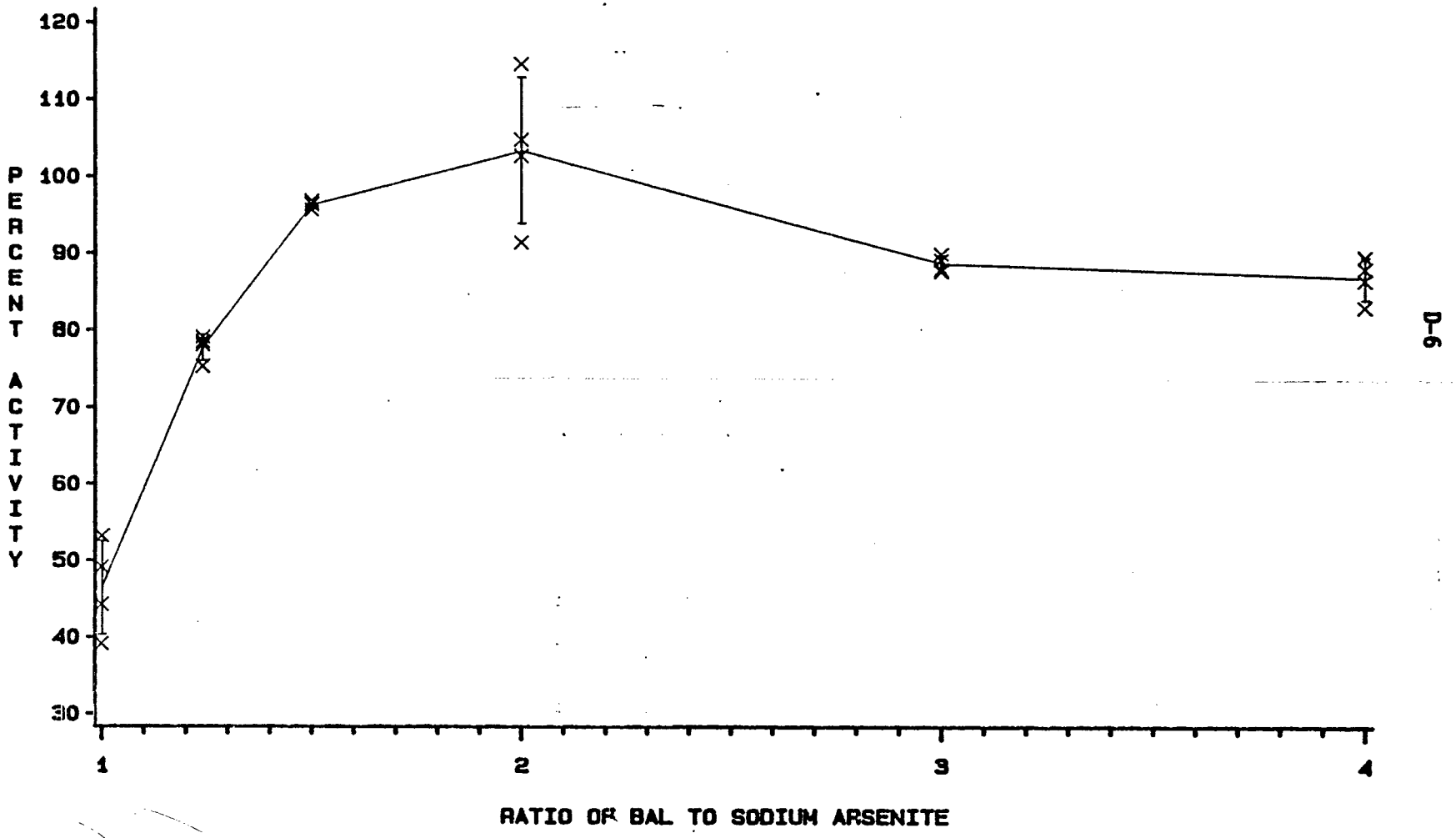


FIGURE 3.5.2. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY SODIUM ARSENITE USING DMPS (X)
WITH ONE STANDARD DEVIATION (-) SHOWN ABOUT THE MEAN AT EACH RATIO

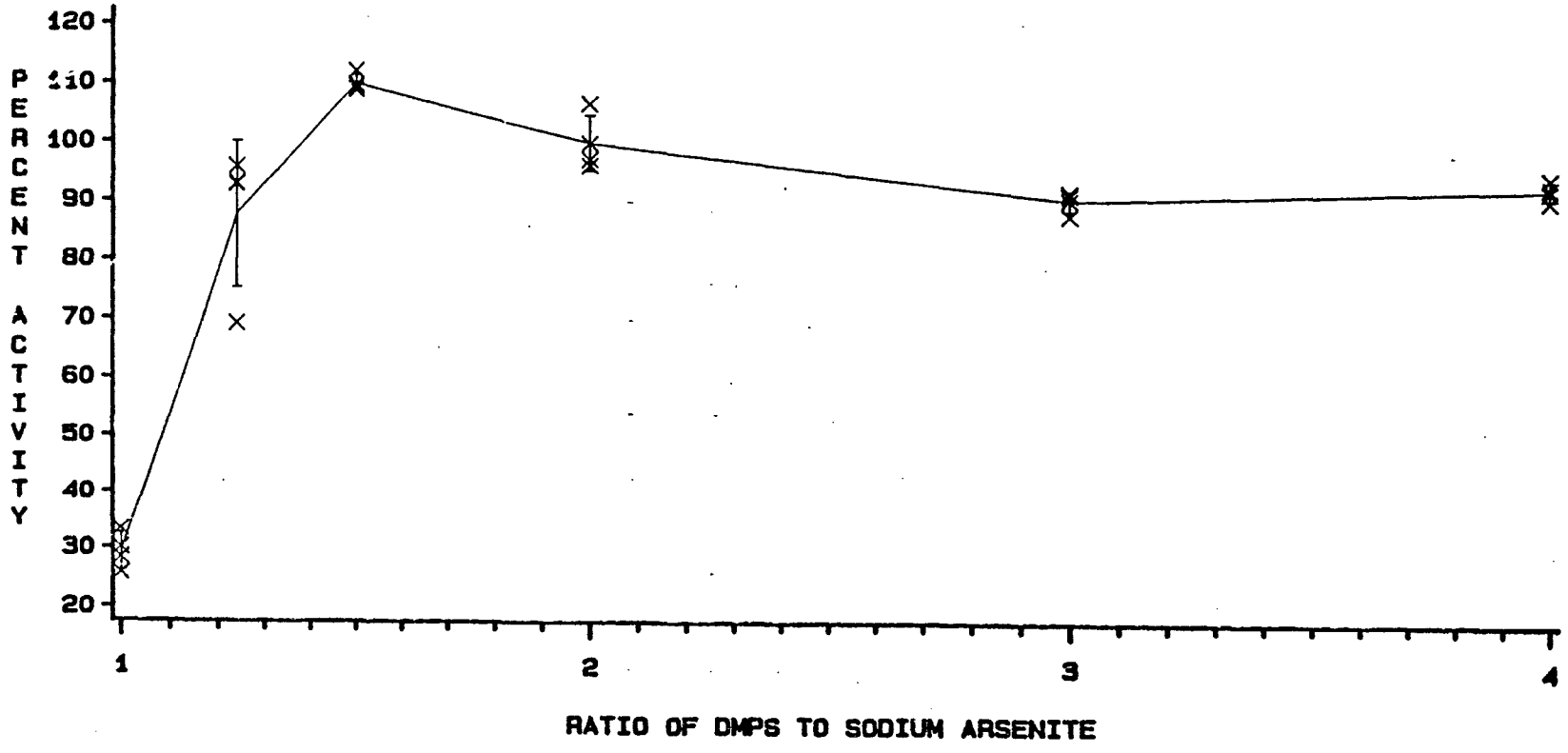


FIGURE 3.5.3. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY SODIUM ARSENITE USING DMSA (X)
WITH ONE STANDARD DEVIATION (-) SHOWN ABOUT THE MEAN AT EACH RATIO

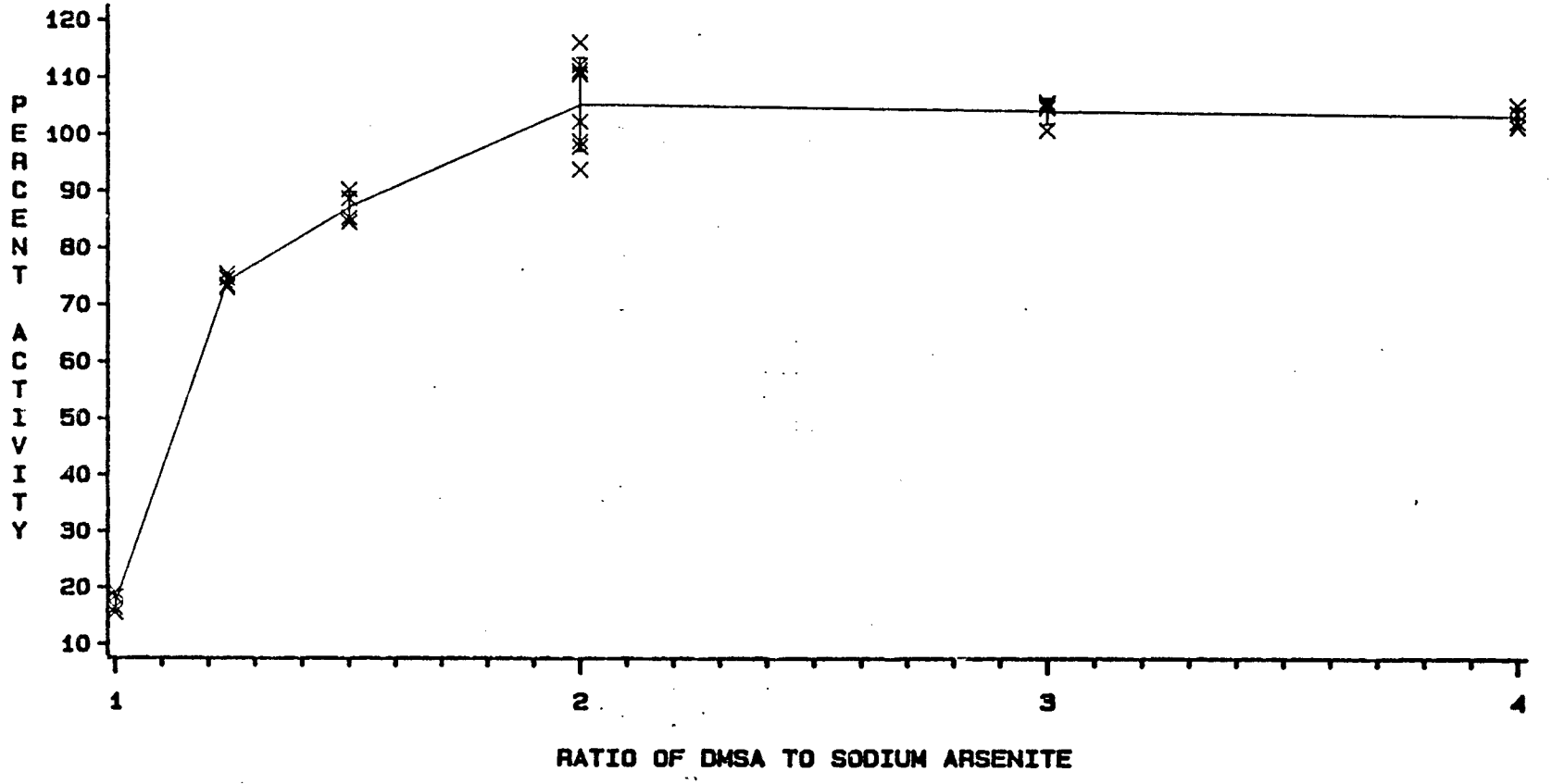


FIGURE 3.5.4. REACTIVATION OF PYRUVATE DEHYDROGENASE INHIBITION BY SODIUM ARSENITE USING BAL, DMPS, OR DMSA

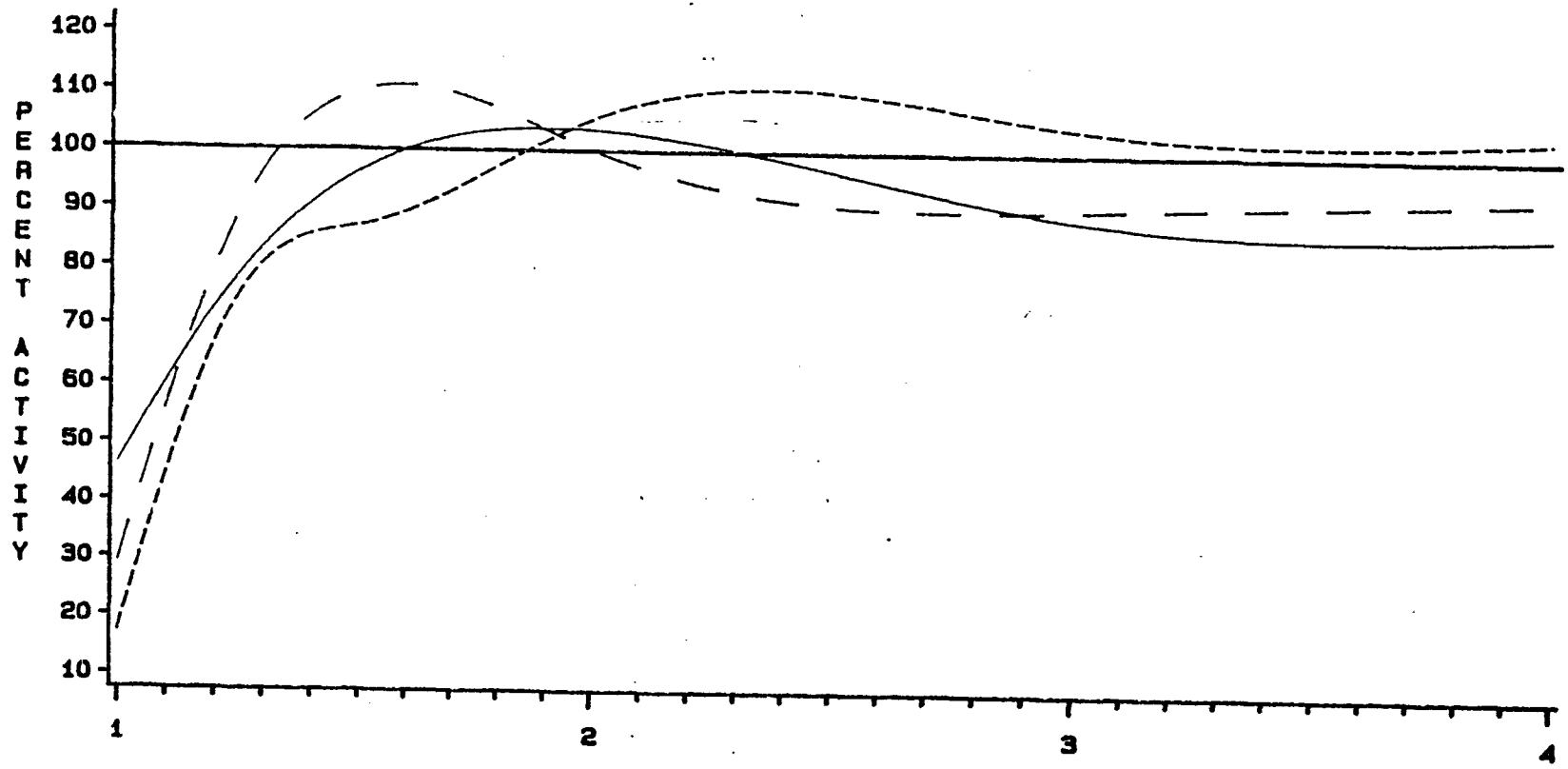


FIGURE 3.6.1. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY LEWISITE USING BAL (X)
WITH ONE STANDARD DEVIATION (-) SHOWN ABOUT THE MEAN AT EACH RATIO

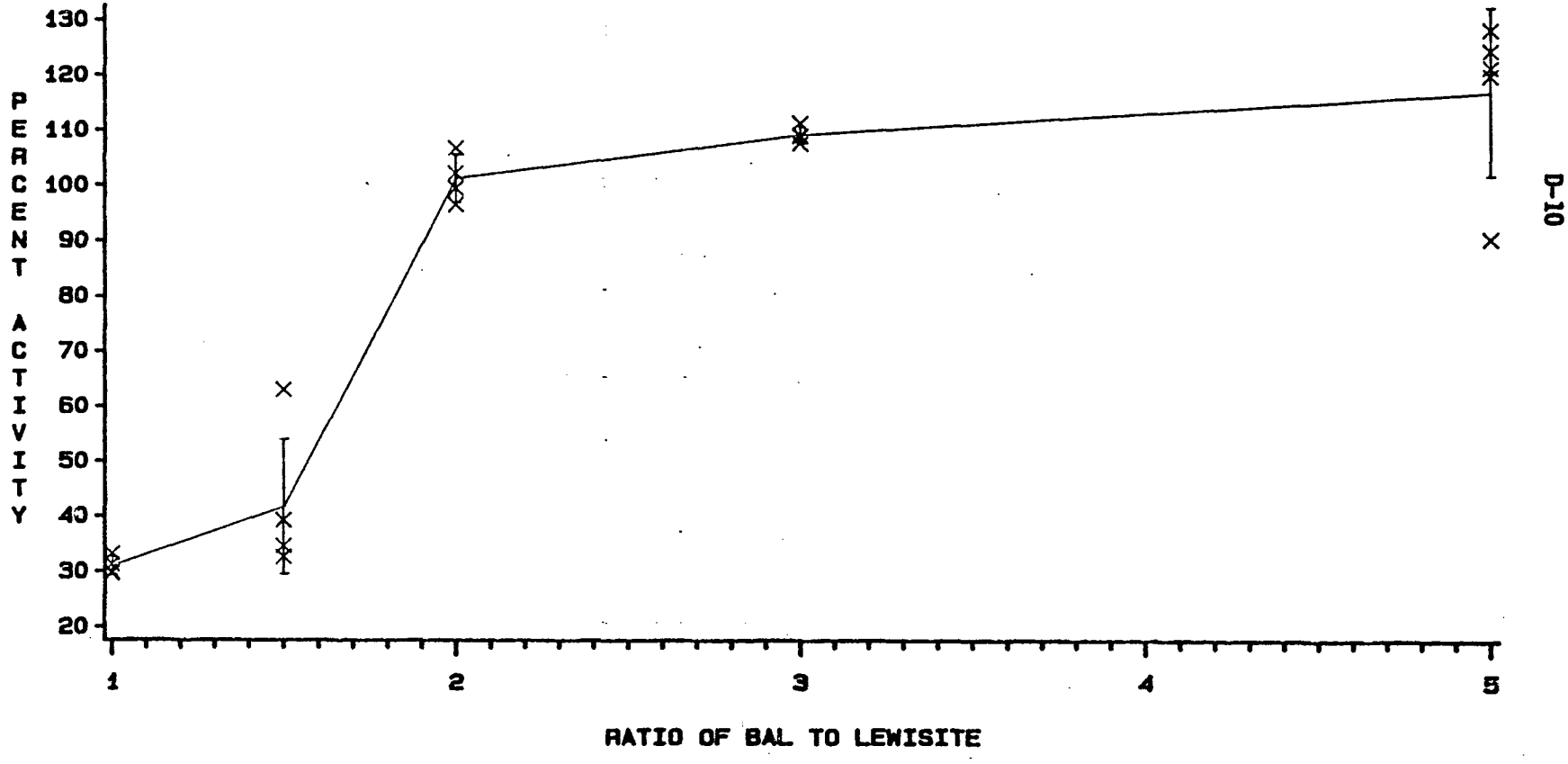


FIGURE 3.6.2. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY LEWISITE USING DMPS (X)
WITH ONE STANDARD DEVIATION (-) SHOWN ABOUT THE MEAN AT EACH RATIO

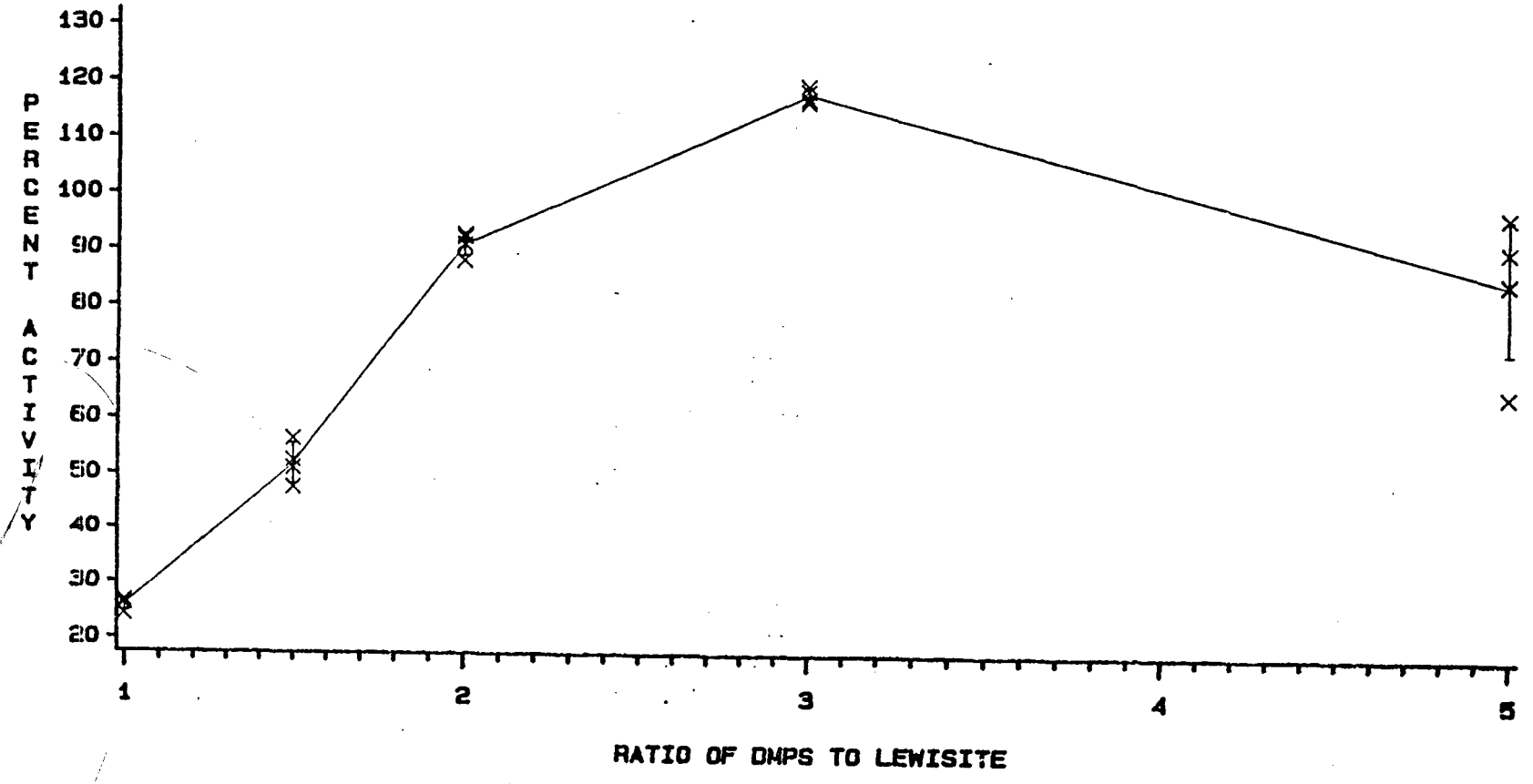


FIGURE 3.6.3. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY LEWISITE USING DMSA (X)
WITH ONE STANDARD DEVIATION (–) SHOWN ABOUT THE MEAN AT EACH RATIO

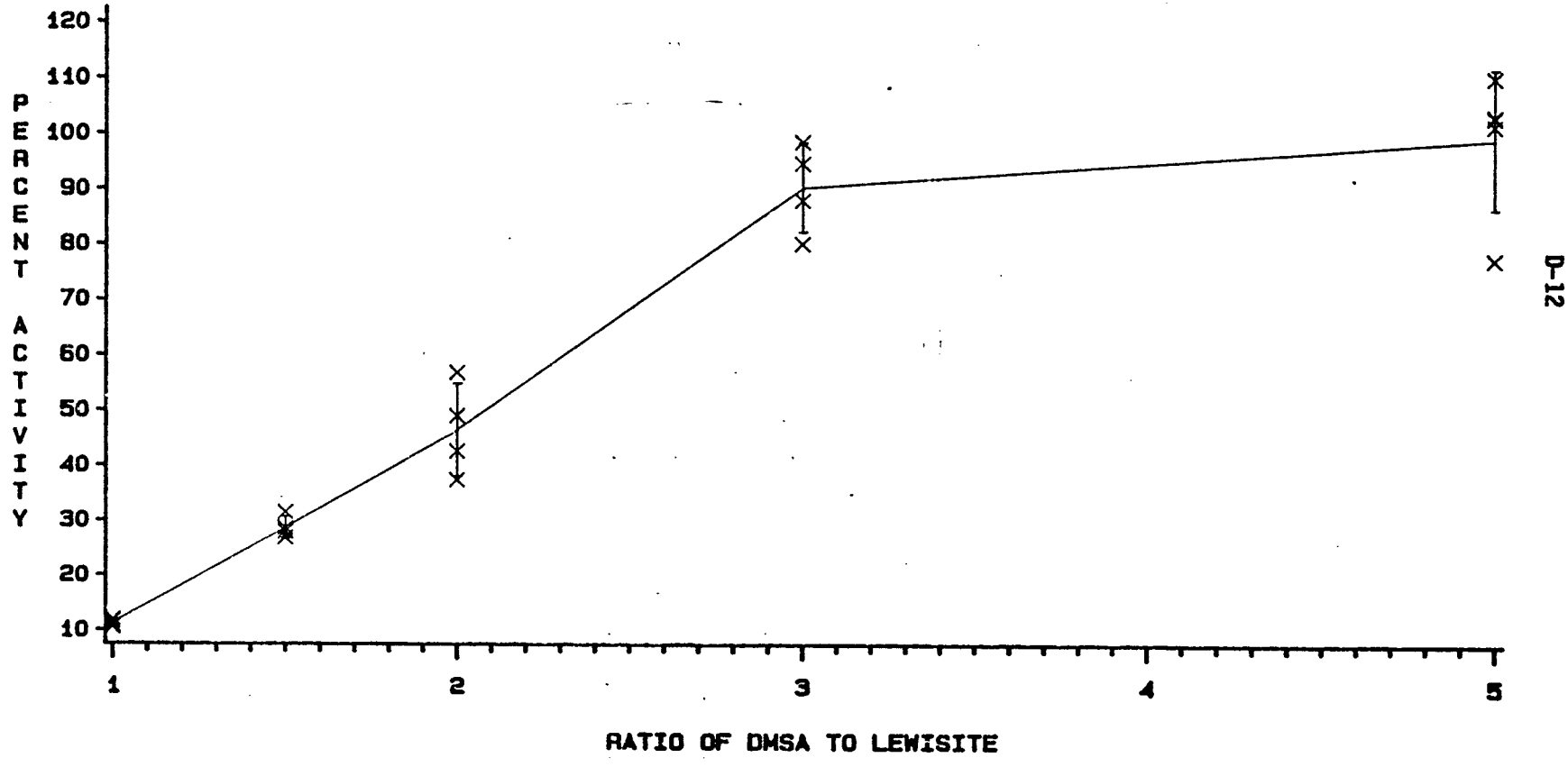


FIGURE 3.6.4. REACTIVATION OF PYRUVATE DEHYDROGENASE INHIBITION BY LEWISITE USING BAL, DMPS, OR DMSA

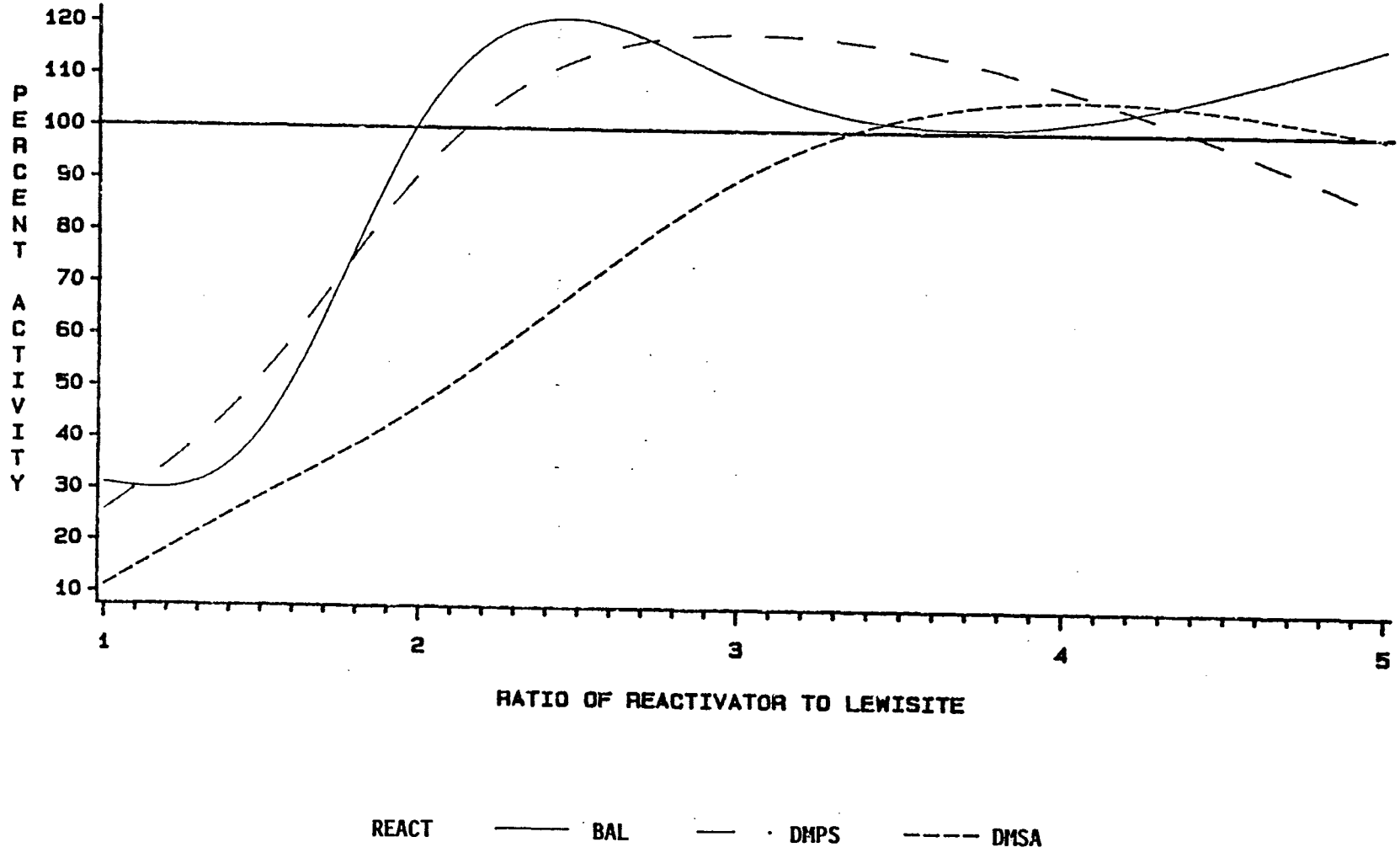


FIGURE 3.7.1. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY CHLOROVINYLARSENOUS ACID USING BAL (X)
WITH ONE STANDARD DEVIATION (-) SHOWN ABOUT THE MEAN AT EACH RATIO

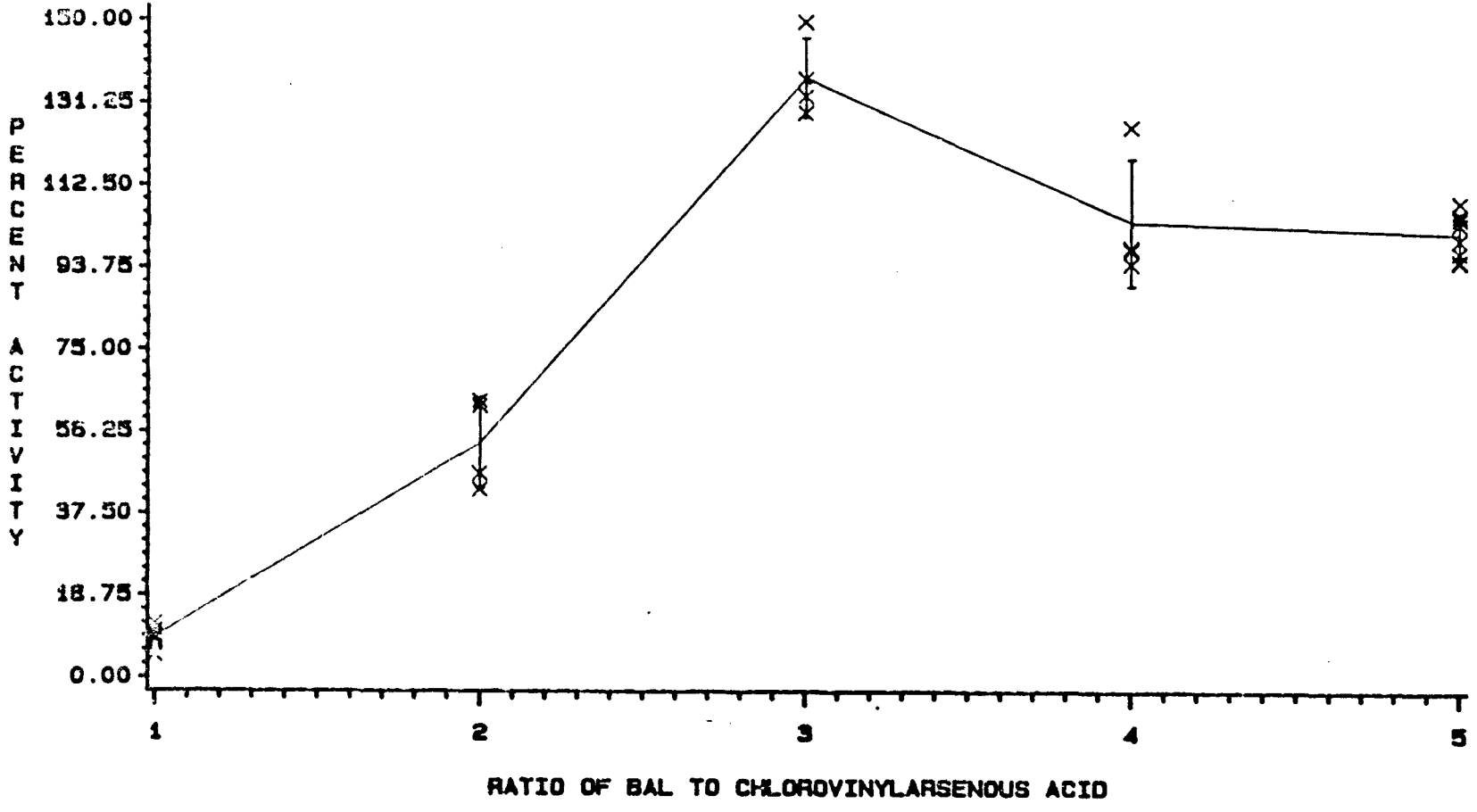


FIGURE 3.7.2. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY CHLOROVINYLARSENOUS ACID USING DMPS (X)
WITH ONE STANDARD DEVIATION (-) SHOWN ABOUT THE MEAN AT EACH RATIO

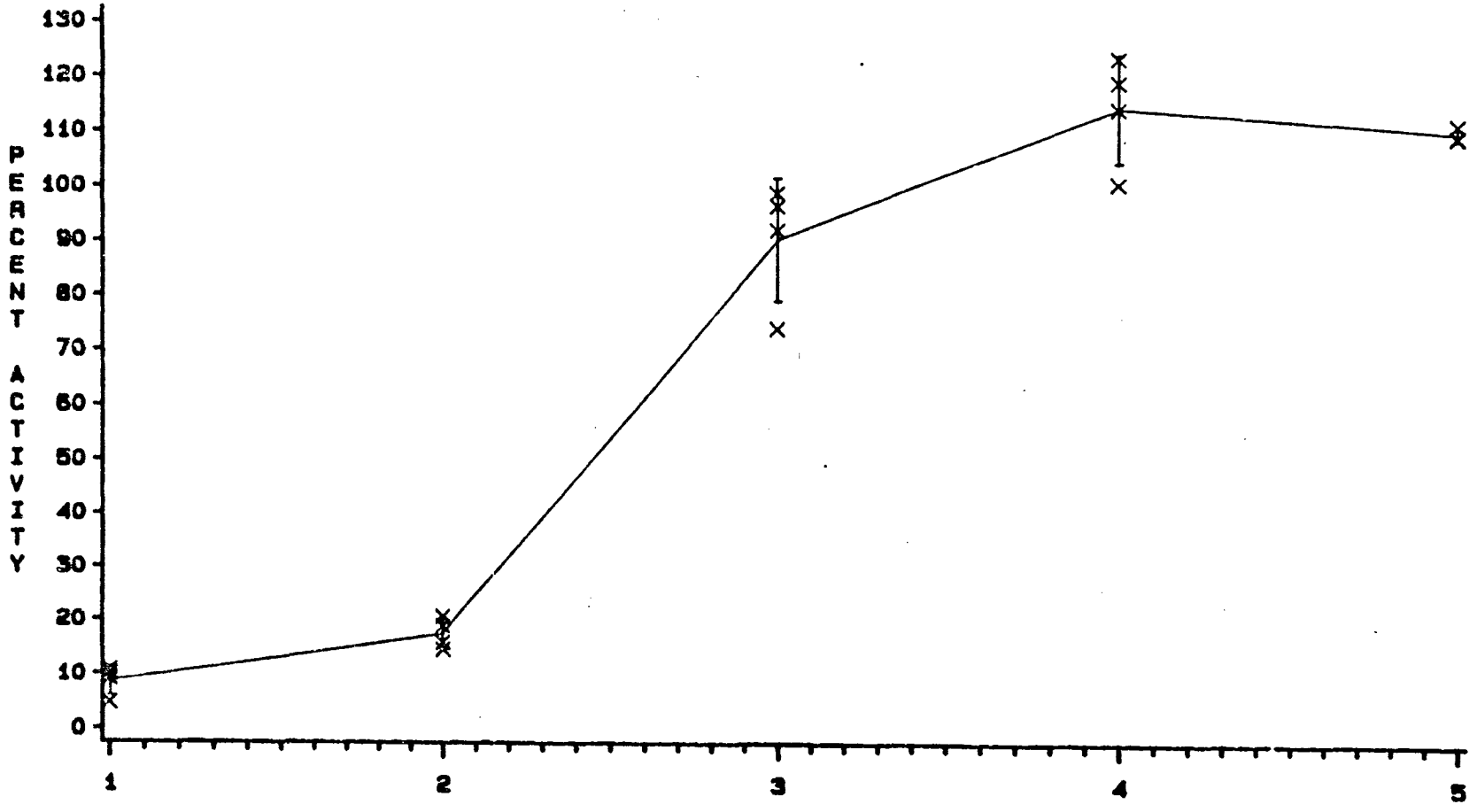


FIGURE 3.7.3. REACTIVATION OF PYRUVATE DEHYDROGENASE

INHIBITION BY CHLOROVINYLARSENOUS ACID USING DMSA (X)
WITH ONE STANDARD DEVIATION (-) SHOWN ABOUT THE MEAN AT EACH RATIO

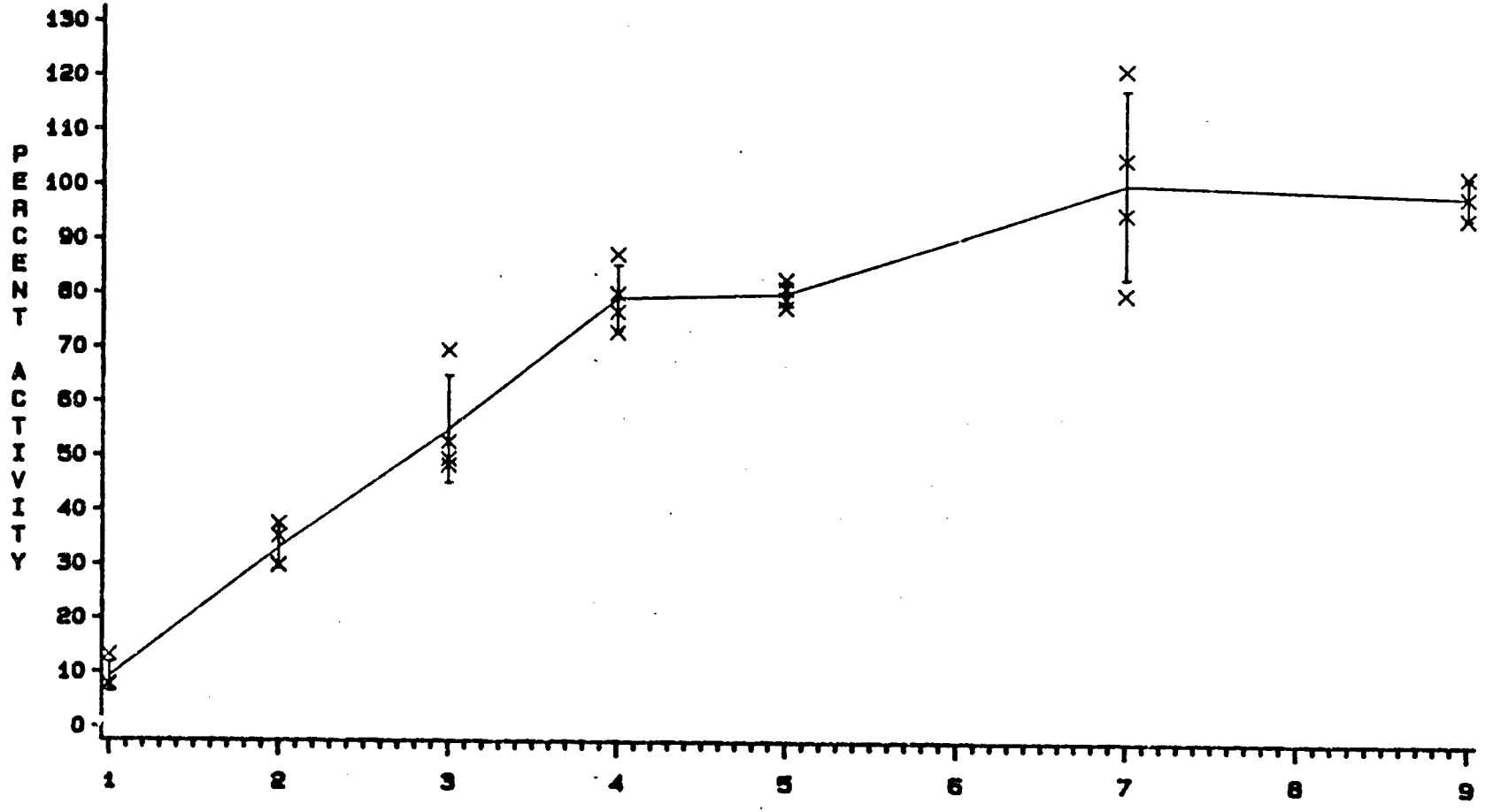
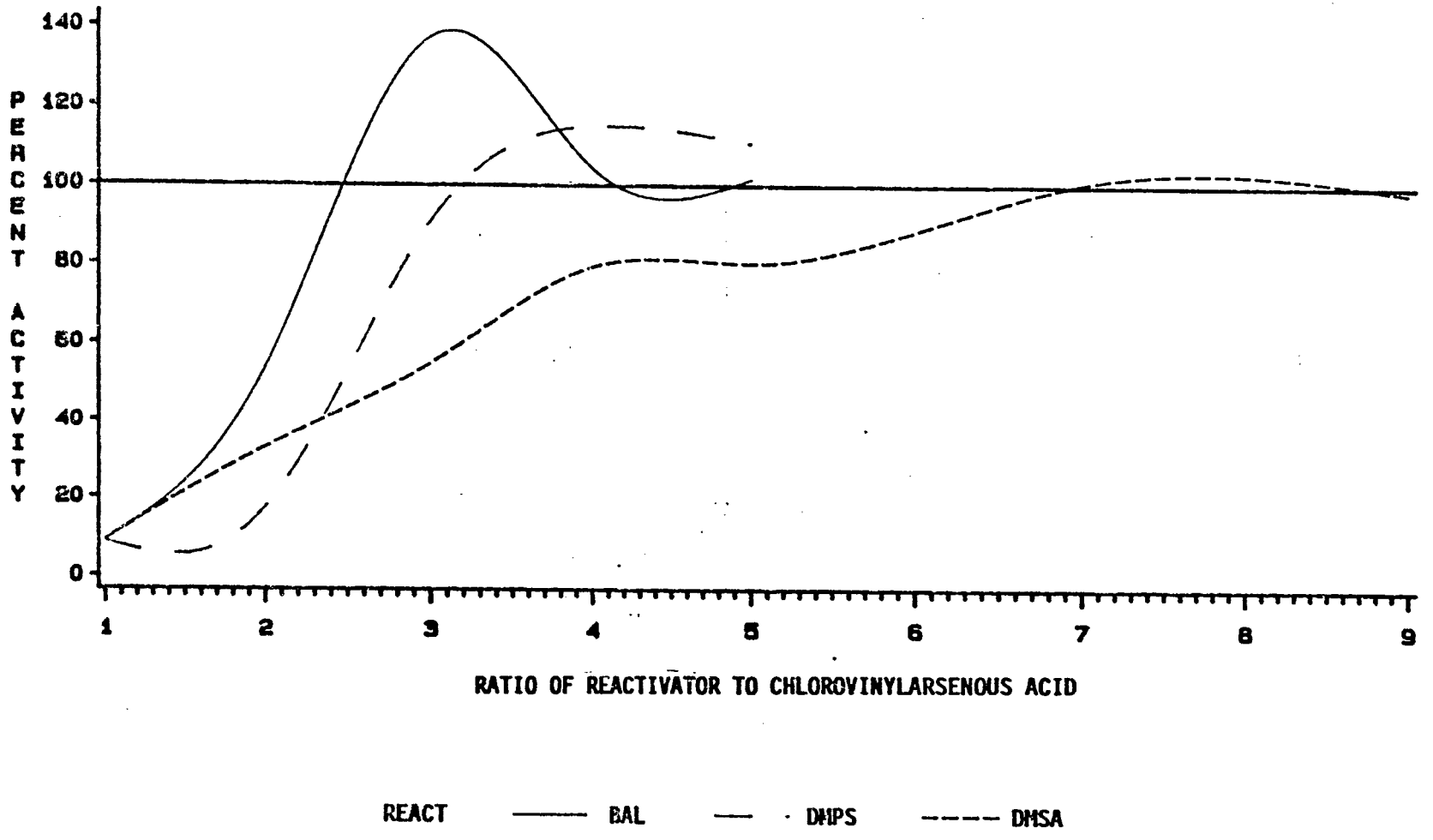


FIGURE 3.7.4. REACTIVATION OF PYRUVATE DEHYDROGENASE INHIBITION BY CHLOROVINYLARSENOUS ACID USING BAL, DMPS, OR DMSA



APPENDIX E

BASIC Computer Program Used to Calculate PDH Activity

```
 0 REM Pyruvate Dehydrogenase Assay Calculation Program
 0 REM by David W. Hobson, Ph.D.
 30 REM Medical Research and Evaluation Facility
 0 REM Battelle, Columbus Laboratories
 0 REM 505 King Avenue, Columbus, Ohio 43201
 60 REM October 1, 1986
 0 CLS
 0 PRINT"PYRUVATE DEHYDROGENASE ASSAY CALCULATION PROGRAM"
 90 PRINT"          by David W. Hobson, Ph.D."
100 PRINT"               (version 1.0)"
 10 PRINT
 20 PRINT"This program calculates the change in absorbance per minute
          the Units/Liter activity, the percent control activity present
          and the percent inhibition produced in samples assayed for "
 30 PRINT"pyruvate dehydrogenase (PDH) complex activity following the
          addition of a test inhibitor, reactivator or reactivator/inhibitor
          combination."
 40 PRINT
 50 PRINT"PDH complex activity determinations are based on the rate of
          conversion of NAD to NADH by dihydrolipoyl dehydrogenase (the
          third enzymatic reaction catalyzed by the complex)."
 60 PRINT
 170 PRINT"One Unit of activity is defined as the conversion of one micromole
          of NAD to NADH per minute by the PDH complex at 30 C and pH 8.1
          in the presence of a saturating amount of coenzyme A."
 180 PRINT:PRINT
 190 INPUT "HIT [return] TO CONTINUE...";D1
 00 CLS
 210 PRINT"PYRUVATE DEHYDROGENASE CALCULATION PROGRAM (CONT.)"
 220 PRINT:PRINT
 30 PRINT:PRINT"The program assumes that you will be entering absorbance data
              for a control sample first, followed by data sets for the"
 240 PRINT"determination of the inhibitory activity of a specified concentration
          of inhibitor."
 50 PRINT
 260 PRINT"It then calculates the percent inhibition and percent activity
          remaining for the enzyme/inhibitor combination relative to the control
          "
 270 PRINT
 280 PRINT"Data entered for enzyme/(inhibitor + reactivator) samples are handled
          as described above, but the control sample used for comparison is an
          enzyme/reactivator only sample instead."
 290 PRINT:PRINT:PRINT
 00 INPUT "HIT [return] TO CONTINUE...";D1
 10 CLS
 320 PRINT"************************************************"
 330 PRINT:PRINT
 40 PRINT" TURN ON THE PRINTER AND PLACE IN ON-LINE MODE."
 350 PRINT" SET PAPER AT THE BEGINNING OF A NEW PAGE..."
 360 PRINT" Consult the printer user's manual for the  "
 70 PRINT" correct procedure for setting the top-of-page"
 30 PRINT" index for your printer."
 390 PRINT:PRINT
 00 PRINT"************************************************"
 10 PRINT:PRINT:PRINT:PRINT
 420 INPUT "HIT [return] TO CONTINUE...";D1
 30 CLS:PRINT:PRINT"SELECT DESIRED ANALYSIS OPTION."
 40 PRINT:PRINT
 450 PRINT"[1] -- Inhibitor Concentration Analysis Only"
 460 PRINT"[2] -- Reactivator Activity Analysis"
```

```
 70 PRINT"[3] -- Determination of Sample PDH Enzyme Activity Only"
480 PRINT"[4] -- Exit Program"
490 PRINT:PRINT
 00 INPUT "Enter Number Corresponding to Analysis Option Desired: ",OP
 10 ON OP GOSUB 520,910,1550,1780
520 REM inhihibitor concentration analysis only
530 SC=0
 40 CLS:PRINT"INHIBITOR CONCENTRATION ANALYSIS ONLY"
550 PRINT:PRINT"This routine performs inhibition calculations on PDH data collec
ted in          accordance with the procedure described in report #123456.
 50 PRINT:PRINT"For each set of samples for a given concentration of inhibitor t
here will be an associated control sample for which its data must be entered fir
st, followed by data for the associated inhibitor samples."
 70 PRINT:PRINT"IMPORTANT NOTE: The sample name for ALL CONTROL samples must beg
n with the      letters CONT or data calculations will be interrupted.
580 GOSUB 2320
590 INPUT"Initials of person entering data? :",TN$
600 LC=0:LPRINT"PYRUVATE DEHYDROGENASE INHIBITOR CONCENTRATION ANALYSIS"
610 LPRINT
620 LPRINT"Date of Analysis: ";DATE$
 30 LPRINT"Data entered by: ";TN$
640 LPRINT
650 LPRINT"Sample Name ";"      PDH ";"     ABSORB  ";"   Activity";"   Inhibiti
n  ";"   r  ";"   n   "
660 LPRINT"            ";"      U/L ";"    (d/min.) ";"       %    ";"       %
   ";"          "
670 LPRINT"------------";"     -----";"   -------- ";"   --------";"   --------
-- ";"   ---  ";" ---"
680 CLS
690 INPUT"Enter sample name (12 letters max.) :",N$
700 IF LEFT$(N$,4)="cont" OR LEFT$(N$,4)="CONT" THEN GOTO 760
710 IF SC=0 THEN PRINT"NO CONTROL DATA HAS BEEN ENTERED!" ELSE GOTO 760
720 INPUT"Do you want to enter data from a new [N] or previous [P] control sampl
e";CD$
730 IF LEFT$(CD$,1)="n" OR LEFT$(CD$,1)="N" GOTO 680
740 CLS:PRINT
750 INPUT"Enter absorbance (d/min.) value for control sample:",SC
760 GOSUB 1790
770 IF LEFT$(N$,4)="cont" OR LEFT$(N$,1)="CONT" THEN SC=S
780 AT=(S/SC)*100
790 IH=(1-(S/SC))*100
800 LPRINT USING "\           \";N$;
810 LPRINT USING"  ####.# ";U;
820 LPRINT USING"    #.####";S;
830 LPRINT USING"       ###.#  ";AT;
840 LPRINT USING"     ###.# ";IH;
850 LPRINT USING"    #.##   ";R;
860 LPRINT N
870 LC=LC+1
880 IF LC=40 GOTO 1480
890 GOTO 680
900 LPRINT CHR$(12):GOTO 620
910 REM Reactivator Activity Analysis
920 SC=0
930 CLS:PRINT"REACTIVATOR ACTIVITY ANALYSIS"
940 PRINT:PRINT"This routine performs reactivator activity calculations on PDH a
ssay data      collected using the PDH complex in vitro screening procedure."
960 PRINT:PRINT"It is assumed that a data set will normally consist, in order, o
r a water      control sample, an inhibitor only sample, a reactivator only sam
ple and several reactivator + inhihibitor samples."
```

```
 30 PRINT:PRINT:PRINT
 40 GOSUB 2320
980 INPUT"Initials of person entering data? :",TN$
990 LC=0:LPRINT"PYRUVATE DEHYDRCGENASE REACTIVATOR ACTIVITY ANALYSIS"
000 LPRINT
1010 LPRINT"Date of Analysis: ";DATE$
1020 LPRINT"Data entered by: ";TN$
030 LPRINT
1040 LPRINT"Sample Name ";"      PDH ";"     ABSORB  ";"   Activity";"   Inhibit
ion  ";"   r  ";"   n   "
050 LPRINT"             ";"      U/L ";"    (d/min.) ";"      %    ";"      %
   ";"          "
1060 LPRINT"------------";"     -----";"    -------- ";"   --------";"   -------
-- ";"   ---  ";" ---"
070 CLS
1080 CLS:PRINT:PRINT
090 INPUT"Enter sample name (12 letters max.) :",N$
100 PRINT:PRINT"1 -- Control"
110 PRINT"2 -- Inhibitor Only"
1120 PRINT"3 -- Reactivator Control"
 30 PRINT"4 -- Reactivator + Inhibitor"
 40 INPUT"Enter number corresponding to sample type :",ST
1150 ST=FIX(ST)
160 IF ST<1 OR ST>4 GOTO 1140
170 IF ST=2 AND SC=0 THEN GOTO 1490
1180 IF ST=3 AND SC=0 THEN GOTO 1490
1190 IF ST<>4 OR SR<>0 THEN GOTO 1280
200 CLS:PRINT"NO REACTIVATOR CONTROL VALUE HAS BEEN ENTERED FOR THIS SAMPLE!"
210 PRINT:PRINT"If no reactivator control data is entered, water control data w
ill be used in   activity/inhibition calculations."
220 INPUT"Do you want to enter data for a reactivator control [Y or N] :";ER$
230 IF LEFT$(ER$,1)="n" OR LEFT$(ER$,1)="N" THEN GOTO 1280
1240 INPUT"Do you want to enter data for a new reactivator control or enter a pr
eviously   determined value? [enter N for new, or P for previous] :",ER1$
 50 IF LEFT$(ER1$,1)="n" OR LEFT$(ER1$,1)="N" GOTO 1080
1260 CLS
1270 INPUT"Enter absorbance (d/min.) value for desired reactivator control:",SR
 80 GOSUB 1790
1290 IF ST=4 AND SR<>0 THEN GOTO 1360
1300 SR=0
310 IF ST=1 THEN SC=S
320 IF ST=3 THEN SR=S
1330 AT=(S/SC)*100
340 IH=(1-(S/SC))*100
 50 GOTO 1380
1360 AT=(S/SR)*100
1370 IH=(1-(S/SR))*100
 80 LPRINT USING "\            \";N$;
390 LPRINT USING"  ####.# ";U;
1400 LPRINT USING"    #.####";S;
410 LPRINT USING"       ###.#  ";AT;
420 LPRINT USING"     ###.# ";IH;
1430 LPRINT USING"    #.##   ";R;
440 LPRINT N
 50 LC=LC+1
1460 IF LC=40 GOTO 1480
1470 GOTO 1070
 80 LPRINT CHR$(12):GOTO 1010
1490 CLS:PRINT"NO CONTROL VALUE HAS BEEN ENTERED YET!"
1500 INPUT"Do you want to enter a value from a previous control [Y or N]",CD$
```

```
1510 IF LEFT$(CD$,1)="n" OR LEFT$(CD$,1)="N" GOTO 1080
1520 CLS
1530 INPUT"Enter absorbance (d/min.) value for previous control: ",SC
1540 GOTO 1280
1550 REM single sample PDH activity determination
1560 CLS:PRINT"DETERMINATION OF SAMPLE PDH ACTIVITY ONLY"
1570 PRINT:PRINT"This routine calculates the PDH activity present in single samp
es prepared     using the PDH complex in vitro screening method. It assumes tha
t samples will be  prepared exactly as described in the above report."
1580 GOSUB 2320
1590 INPUT"Initials of person entering data? :",TN$
1600 LC=0:LPRINT"SINGLE SAMPLE PYRUVATE DEHYDROGENASE ACTIVITY DETERMINATION"
1610 LPRINT"Date of Analysis ";DATE$
1620 LPRINT"Data Entered by ";TN$
1630 LPRINT
1640 LPRINT"Sample Name","PDH (U/L)","abs. (d/min.)","   (r)   ","n"
1650 LPRINT"___________","_________","_____________","_________","_"
1660 CLS
1670 INPUT"Enter sample name (12 letters max.) :",N$
1680 GOSUB 1790
1690 LPRINT USING "\          \";N$;
1700 LPRINT USING "    ####.#          ";U;
1710 LPRINT USING "#.####      ";S;
1720 LPRINT USING "#.###       ";R;
1730 LPRINT N
1740 LC=LC+1
1750 IF LC=40 GOTO 1770
1760 GOTO 1660
1770 LPRINT CHR$(12):GOTO 1610
1780 CLS:END
1790 REM calculation of sample change in absorbance per minute
1800 CLS:PRINT"Sample Name = ";N$
1810 PRINT
1820 INPUT"How many absorbance values do you want to enter for this sample";N
1830 N=FIX(N)
1840 IF N<1 THEN 1800
1850 DIM X(N),Y(N)
1860 Z1=0:Z2=0:Z3=0:Z4=0:Z5=0
1870 FOR J=1 TO N
1880 PRINT CHR$(7)
1890 PRINT"Enter time value #";J;" in minutes."
1900 INPUT X(J)
1910 PRINT"Enter corresponding absorbance value for ";X(J);" minutes."
1920 INPUT Y(J)
1930 CLS
1940 NEXT J
1950 CLS
1960 PRINT"Data entered for the sample named ";N$;" is as follows"
1970 PRINT"Value #";"   ";"Time (min.)";"    ";"Absorbance"
1980 PRINT"-------";"   ";"-----------";"    ";"----------"
1990 FOR K=1 TO N
2000 PRINT" ";K;"       ";
2010 PRINT USING"##.##";X(K);
2020 PRINT USING"          #.######";Y(K)
2030 NEXT K
2040 PRINT
2050 FOR I=440 TO 880 STEP 40
2060 SOUND I, .5
2070 NEXT I
2080 INPUT"Is the name for this sample correct [Y or N]: ";D$
```

```
2090 IF LEFT$(D$,1)="y" OR LEFT$(D$,1)="Y" GOTO 2120
2100 INPUT"Enter new sample name (12 letters max.) :",N$
2110 GOTO 1950
2120 INPUT"Is this data set correct (Y or N)";Q$
2130 IF LEFT$(Q$,1)="y" OR LEFT$(Q$,1)="Y" GOTO 2180
2140 INPUT"Enter the number of a data point you wish to correct: ",L
2150 INPUT"Enter new time value:",X(L)
2160 INPUT"Enter new absorbance value:",Y(L)
2170 CLS:GOTO 1960
2180 FOR I=1 TO N
2190 Z1=Z1+X(I)
2200 Z2=Z2+Y(I)
2210 Z3=Z3+X(I)*Y(I)
2220 Z4=Z4+X(I)^2
2230 Z5=Z5+Y(I)^2
2240 NEXT I
2250 Z6=N*Z4-Z1^2:Z7=N*Z5-Z2^2
2260 R=(N*Z3-Z1*Z2)/SQR(Z6*Z7)
2270 S=(N*Z3-Z1*Z2)/(N*Z4-Z1^2)
2280 REM conversion of abs. (d/min) to U/L
2290 U=S*3360.129
2300 ERASE X,Y
2310 RETURN
2320 PRINT:PRINT
2330 INPUT"HIT [return] TO CONTINUE OR [M] TO SELECT ANOTHER ANALYSIS OPTION: ",
D1$
2340 IF LEFT$(D1$,1)="m" OR LEFT$(D1$,1)="M" GOTO 430
2350 CLS
2360 RETURN
```

APPENDIX F

Methods for the Preparation of Dilute CVAA and L Solutions

METHOD FOR PREPARATION OF CHLOROVINYLARSENOUS ACID SOLUTION*

Prepared by: Dr. Millard M. Mershon

6 October 1986

1. Measure 0.1 mℓ of neat Lewisite (L) in an accurate glass pipette or glass syringe. Accuracy may be validated by weighing the volume of L (density 1.88 g/mℓ at 25 C).

   Note: L reacts with metals, therefore, microliter (μℓ) or milliliter (mℓ) syringes with metal fittings should be avoided. (Special platinum fittings resist L).

2. Deliver L into a 100 mℓ volumetric flask containing 1.0 mℓ of 0.1N HCl and 50 mℓ of deionized/distilled water.

3. Stopper volumetric flask and place on suitable shaker for agitation overnight to completely dissolve the milky appearing globules of chlorovinylarsenoxide that are formed during L hydrolysis. Chlorovinylarsenoxide is very slightly soluble but slowly solubilizes to form chlorovinylarsenous acid solution (CVAA).

4. Decant the CVAA solution into a 100 mL beaker and add 0.1N NaOH solution dropwise to adjust pH to 5.8, if the product will be used with enzyme or cellular preparations. If so, replace adjusted solution into volumetric flask, rinse beaker with saline in several aliquots, and bring flask to 100 volumes.**

5. pH adjustment or saline may be omitted and water may be used to complete 100 volumes, if appropriate for analytical procedures or other use.

*All required safety and surety precautions will be observed.
**A 1:1000 solution contains 1.88 mg/mℓ, less than the 2 mg/mℓ limit for dilute agent regulations.

METHOD FOR PREPARATION OF DILUTE LEWISITE SOLUTION IN pH 8 TRIS BUFFER

Prepared by Dr. David W. Hobson

6 October 1986

1. For a final Lewisite (L) concentration of 20 mM (4.55 grams/liter [g/ℓ]), prepare the following dilution:

   .20732 g/mM x 20 mM/ℓ x 1 mℓ/1.88 g x 1 mℓ/.91 mℓ
   = mℓ (L)/ℓ (stock purity)

2. For 100 mℓ of 20 mM L:

   a. Add 0.242 mℓ stock L to 80 mℓ Tris buffer (pH = 8, the same buffer concentration used in the PDH complex assay).

   b. Adjust back to pH = 8 with 0.1N NaOH after 30 min.

   c. Let stand overnight.

   d. Adjust to pH = 8 with 0.1N NaOH, quantitatively transfer to a 100 mℓ volumetric flask and bring total volume to 100 mℓ with pH = 8 Tris buffer.